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OBITUARY NOTICE.

WALTER MORLEY FLETCHER.

(1873–1933.)

THE death of Sir Walter Fletcher, Secretary of the Medical Research Council, on June 7th, in the sixtieth year of his age and the nineteenth of his tenure of an office he had made great, removed with tragic unexpectedness a public servant who by sheer merit had raised himself to be the trusted captain and apologist of a great host of workers striving in their many different ways and for the most part in the seclusion of the laboratory for the advancement of scientific medicine.

These now mourn the loss of a friend. Medical research, as the late Sir Walter never ceased to emphasise in many a weighty and admirably drafted pronouncement, calls to its service the labour corps of many ancillary sciences and from none perhaps does it expect more help in the solution of its increasingly complicated problems than from Biochemistry.

To the members of the Biochemical Society, therefore, of which Fletcher was an original member, his loss is a particularly severe one, in that his appreciation of the part that Biochemistry has to play in the general advancement of medical science came from his own discipline and distinguished performance in physiological chemistry throughout a period of nearly twenty years.

Undoubtedly Fletcher would have called himself a physiologist first and foremost, but, so far as concerned the particular problem to which his laboratory life was dedicated, he had the clearest vision of its further illumination when helped on its way by modern biophysics and modern biochemistry—a vision that has more than materialised. The many appreciations of the late Sir Walter which have already appeared and their varied sources are eloquent testimony to the numerous links he had forged between the laboratory worker and the outside world of affairs. His task may be said, indeed, to have been to explain the day's work—the *ἔργα καὶ ἡμέραι*—of the laboratory to a non-scientific and not always too appreciative public, a public, however, which paid the piper and might even, if unwisely guided, call the tune. Fletcher was the conductor in fact of a great orchestra of many and strange instruments, and it was his business to harmonise and co-ordinate the tunes of each to the greater glory of medical progress. "Instruments," I venture to think, would have pleased him. It was a favourite notion of his that disciplines should be divided not according to their matter but according to their methods and that the latter alone had in them the seeds of universal value.

What then were Sir Walter's antecedents and how was it that he, a devoted man of science yet not unfamiliar with affairs, resigned his place in the orchestra to become its conductor when the call came to him just before the outbreak of the great war?

Fletcher began his academic career in 1891 when he entered Trinity College Cambridge. Three years later he graduated with first class honours in the Natural Science Tripos, and from that date for nearly twenty years the Cambridge School of Physiology was his workroom and Trinity College, of which he was elected

a Fellow in 1897 and to whose affairs he was devoted, his real home. His clinical training in medicine he took at Bart.'s and in 1900 he obtained his Cambridge M.B.

Throughout his whole Cambridge career Fletcher's immediate scientific interests were centred almost exclusively in the problem of muscle respiration, an important field of enquiry in which, at the outset of his work, little or no certain knowledge was available. Between 1898 and 1914 he contributed to the *Journal of Physiology* a valuable series of papers on this subject, including two in collaboration, respectively, with Hopkins and G. M. Brown. These papers definitely established Fletcher's reputation as a physiologist of distinction. His first contribution in 1898 on the survival respiration of muscle was a substantial one of nearly a hundred closely reasoned pages, and fully documented, the elaborate apparatus of which he made use for continuous estimations of CO_2 being a modification of that devised by F. F. Blackman for research on gaseous exchange in plant leaves. The work was carried out during his tenure of the Coutts-Trotter Studentship and was communicated to the 4th International Physiological Congress which met in Cambridge in August of that year.

The normal curve of CO_2 discharge from excised frog muscle was fully worked out and the effect thereon of various poisons and temperature changes. Of fundamental importance was his observation that in an excised muscle which has been made to contract, there was no accompanying increase in the rate of CO_2 discharge. Throughout this work Fletcher took pains to exclude from consideration late yields of CO_2 arising from putrefactive changes in the muscle.

In his second and third papers in 1902 another important stage was reached when, in investigating the influence of oxygen on the survival respiration of muscle, he showed that loss of irritability in a surviving muscle is greatly delayed by an abundant supply of oxygen.

In 1904 he recorded the fact that exposure of a fatigued muscle to an atmosphere of oxygen restored to it, in a marked degree, the osmotic properties of resting muscle.

In 1907, in collaboration with Hopkins, appeared a most important contribution to the biochemistry of muscle showing that contraction of excised muscle is regularly accompanied by an increase of lactic acid and that, if the fatigued muscle is placed in oxygen, the lactic acid already formed disappears. The paper contains also the technical details of a new colour reaction for lactic acid devised by Hopkins for this work.

Later papers in 1911, 1913 and 1914 (the last in collaboration with G. M. Brown) continue the discussion of lactic acid production particularly in connection with its alleged formation during autolysis and its formation in mammalian muscle.

Fletcher's work and that of his collaborator Hopkins laid the foundations on which was built the elaborate treatment on thermodynamic lines of muscular action by Hill and Meyerhof. An analysis of its main conclusions will be found in the Croonian Lecture before the Royal Society by Fletcher and Hopkins in 1915 (*Proc. Roy. Soc. Lond.* 1917, B **89**, 444) while critical reviews by A. V. Hill of the relations existing between heat production in muscle and the underlying chemical processes will be found in the *Ergeb. d. Phys.* 1916, **15**, 340 and in *Phys. Rev.* 1922, **2**, 310.

Writing in 1923 (*Nature*, July 14th, Suppl.), A. V. Hill ascribed nearly all the recent advances in muscle physiology to the study of the phenomena of fatigue in muscle. Recovery from fatigue occurred apparently only in the presence of oxygen, while the lactic acid, which was found by Fletcher and

Hopkins to increase by exercise, was diminished or abolished by recovery in the presence of oxygen. Further work on the problem demanded a new technique for correlating the heat produced with the observed chemical phenomena. This was supplied by the delicate thermoelectric apparatus devised by Hill, and by its aid it has been possible to build up what is a completely new chapter in muscle physiology—the thermodynamics of muscular work. The mysterious lactic acid is now known from estimation of the heat evolved in contraction to be derived from glycogen. In recovery the lactic acid is rebuilt into the glycogen from which it was derived except for about one-sixth of it which is oxidised to provide energy for the restoration. The essential point is that contraction and discharge of lactic acid are anaerobic processes and that oxidation is concerned only with the phenomena of recuperation.

It has been said that Fletcher's soul was not fully and entirely satisfied by the strivings and triumphs of laboratory toil and that he sought a wider arena in which his intellectual versatility and his flair for managing might have full play. Possibly he felt that after 18 years' devotion to one single problem of high physiological importance, he could safely say that he had rounded it off quite satisfactorily according to his lights and that he might now pass on the burden of its further pursuit to workers of other disciplines, biophysics, molecular physics, enzyme chemistry and what not. The call came to him on July 1st, 1914, to take up the duties of Secretary of the Medical Research Committee founded in 1913 as part of the provisions of the National Insurance Act of 1911. From the wide scope of the Committee's reference, and its own views with regard to the extension of the term "Medical Research" as part of a national scheme, it was quite clear that, in the search for the ideal secretary, no ordinary person of narrow views and interests was likely to be successful in guiding its destiny. Fletcher proved himself no ordinary person.

This is how the Committee outlined their conception of research.

"The object of the research is the extension of new knowledge with the view of increasing our powers of preserving health and preventing or combating disease. But otherwise than that this is to be the guiding aim, the actual field of research is not limited and is to be wide enough to include, so far as may from time to time be found desirable, all researches bearing on health and disease whether or not such researches have any direct or immediate bearing on any particular disease or class of diseases provided that they are judged to be useful in promoting the attainment of the above object."

How well Fletcher and his Committee of changing personnel succeeded in translating into administrative practice the wide conception of research contained in this carefully drafted statement and how deftly he defended, when necessity arose, the more subtle and perhaps not too readily appreciated implications of its later phrases, are matters of history.

It is the history in fact of a highly fertile period of medical research in this country and the empire, which owes much of its success to Fletcher's gifts of co-ordination and the personal and friendly terms on which he invariably met the numerous workers and grantees in the vast organisation of which he held the threads.

This is not the place to record in detail the landmarks of Fletcher's 18 years' secretaryship—the pooling of the forces he wielded, at the very outset of his career, to meet the medical emergencies of war, the happily engineered emergence of the Medical Research Committee as the Medical Research Council with a new Charter and holding office with greater freedom and responsibility under the Privy Council, the centralisation at the National Institute, Hampstead, of

a permanent nucleus of experts in many fields of medical enquiry, and, as the years passed, the numerous opportunities seized by Fletcher to promote his one great ambition, to see the health of man and beast cared for and studied not only as a national and imperial charge but as the natural and fitting obligation of every right-thinking individual. One may fittingly refer here, however, to his many efforts in the cause of biochemical research and pay tribute to his many successful interventions in securing financial help for what are now active centres of research not only in biochemistry but also in nutritional studies. These centres have greatly enriched the resources at the Empire's disposal for attacking effectively the many problems that concern the health of its human and animal populations. Nutrition studies and all that these imply were very dear to Fletcher. Possibly the last reasoned article from his facile pen was a contribution to the new journal, *Nutrition Abstracts and Reviews* (1932), on the urgency of nutritional studies. There, in commending the new journal to its readers, he referred to the range and variety of interests served by advances in nutritional science and to the fact that no visible limits can be set to them. A perusal of the table of contents of any number of this new journal is indeed sufficient to indicate the far-reaching character of nutrition problems and goes far to establish a real truth underlying the adage "Der Mensch ist was er isst." Fletcher in his rôle of the complete advocate would have added "and beast too."

By his very position Fletcher could feel the pulse of medical progress at many points and generally with much acumen. So far as biochemical and nutritional science is concerned, its devotees owe him much for the sympathetic and always helpful interest he invariably took in their special problems.

Lastly, a glimpse of Fletcher in the midst of his daily office routine, interviewing friends and strangers, arranging committees and often presiding over them, translating draft reports, resolutions and preambles into the excellent English on which he justly prided himself and through it all, striving to secure action and effective action. It has amazed me to watch him preside, it might be, over a conference on poliomyelitis and to see him display his wonderful flair for ordered discussion and his real understanding of the subject in hand. His versatility was not that of the briefed barrister. Truly Fletcher's scientific discipline stood him well. Eighteen annual reports of his Council will inform the medical historian of the future what Fletcher did and what he strove to do to further the work and influence of an organisation which has abundantly justified its national character, but, without their aid, his memory will long remain green in the hearts of those who knew and loved and admired him.

J. C. G. L.

CLXXX. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

III. (*a*) THE EFFECT OF INJECTIONS OF ANTERIOR PITUITARY EXTRACTS ON URINARY CREATININE IN NORMAL AND CASTRATED RABBITS.

(*b*) THE TOLERANCE OF NORMAL AND CASTRATED ANIMALS TO INJECTED CREATINE.

By ISIDORE SCHRIRE and HARRY ZWARENSTEIN.
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(*Received June 16th, 1933.*)

The evidence for the existence of an endocrine relationship between the testes and creatinine metabolism was presented in the first two papers of this series [Schrire and Zwarenstein, 1932, 1, 2]. This conclusion is supported by the observations of Vedel, Peuch and Lang [1926] who describe the case of a man of 56 who had been castrated and showed regression of the secondary sexual characters. His creatine and creatinine excretion was subsequently investigated. No creatine was excreted but the amount of creatinine in the urine was 2·8 g. per litre. According to Folin [1905] the concentration of creatinine in normal human urine varies from 0·61 to 2·0 g. per litre with an average of 1·08. In the second paper cited above it was suggested that the increased elimination of creatinine after castration was primarily due to hyperactivity of the anterior lobe of the pituitary, consequently injections of anterior lobe extracts should cause an increase in the excretion of creatinine. In this paper evidence is presented in support of the hypothesis. As a contribution to the more detailed analysis of the problem the effect of injections of creatine into normal and castrated animals was also investigated.

Experimental.

Details in regard to caging, feeding, collection and analysis of urine were given in the first paper of this series. In Table I the dates refer to the first of a number of 24-hour periods. The mean and the standard deviation of the daily

Table I. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)	No. of 24-hour periods
A. Pre-periods:					
12. iv. 32	92±5·2	76±5·8	89±3·2	—	9
7. v. 32	93±5·5	93±3·5	91±2·4	—	5
17. vi. 32	97±3·1	87±4·3	79±1·0	86±4·1	4
		Nos. 2 and 3 castrated 21. vi. 32.			
B. Post-castration periods:					
12. xii. 32	96±7·4	120±1·9	123±11·6	84±8·3	5
27. xii. 32	98±2·5	116±9·1	119±10·0	91±3·2	4
16. i. 33	95±3·7	111±2·6	122± 5·2	98±7·0	4

creatinine output in mg. are given for each series of 24-hour periods. In all Tables rabbits Nos. 1 and 4 are the normal animals and Nos. 2 and 3 the castrated animals.

(*a*) Injection of anterior lobe extracts.

Parke-Davis antuitrin was used and also an extract of sheep anterior lobe prepared according to Bellerby's method [1933]. As a control an extract of brain prepared by the same method was injected. These were all injected subcutaneously.

Table II. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
16. i. 33	92	109	120	100
17. i. 33	96	109	114	92
18. i. 33	92	*109	126	*92
19. i. 33	101	115	127	109
20. i. 33	105	120	134	114
21. i. 33	100	109	120	93
22. i. 33	101	120	126	85
23. i. 33	100	†120	†126	†92
24. i. 33	92	120	126	112
25. i. 33	86	109	114	92
26. i. 33	100	120	120	92
27. i. 33	100	114	133	90

* On Jan. 18 No. 2 injected with 2 cc. brain extract, No. 4 with 2 cc. anterior lobe extract.
† On Jan. 23 No. 2 injected with 2 cc. anterior lobe extract, No. 3 with 2 cc. brain extract and No. 4 with 2 cc. antuitrin.

Table III. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
28. ii. 33	101	120	114	84
1. iii. 33	100	120	109	86
2. iii. 33	100	*109	*130	*90
3. iii. 33	104	120	123	110
4. iii. 33	98	110	109	80
5. iii. 33	96	109	121	96
6. iii. 33	93	†114	126	†80
7. iii. 33	100	109	120	106
8. iii. 33	104	120	130	109
9. iii. 33	92	109	120	92
10. iii. 33	93	109	130	88

* On March 2 Nos. 2, 3 and 4 each injected with 3 cc. antuitrin.
† On March 6 Nos. 2 and 4 each injected with 3 cc. antuitrin.

Table IV. *Daily urinary creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
10. iv. 33	96	120	133	100
11. iv. 33	96	126	140	88
12. iv. 33	*92	*120	*136	*96
13. iv. 33	125	134	140	96
14. iv. 33	100	122	125	96
15. iv. 33	100	133	126	96
16. iv. 33	†100	†126	133	†98
17. iv. 33	133	120	133	126
18. iv. 33	114	122	126	105
19. iv. 33	100	120	126	88
20. iv. 33	100	120	135	96

* On April 12 Nos. 1 and 3 each injected with 3 cc. anterior lobe extract; Nos. 2 and 4 each injected with 3 cc. brain extract.
† On April 16 Nos. 1, 2 and 4 each injected with 3·5 cc. anterior lobe extract.

The following conclusions can be drawn from the data presented above.

(*a*) Injection of antuitrin or of anterior lobe extract into normal animals caused an increased excretion of creatinine. The figures suggest that the increase bore a constant relation to the dosage. This is seen in the following table. The figures in brackets refer to the number of the animal. The average weight of rabbit No. 1 during the injection experiments detailed above was 2·74 kg. and of rabbit No. 4, 2·34 kg.

	Antuitrin		Anterior lobe extract		
Amount injected	2 cc.	3 cc.	2 cc.	3 cc.	3·5 cc.
Percentage increases	22 (4)	26 (4)	22 (4)	30 (1)	33 (1)
	—	28 (4)	—	—	30 (4)

In all cases the rise occurred on the day following injection, persisted in a few cases during the next day and in all cases rapidly returned to the normal level.

(*b*) Injection of antuitrin or of anterior lobe extract had no effect on the high creatinine level in the castrated animals.

(*c*) Brain extract had no effect either in the normal or in the castrated animals.

Discussion.

Braier [1931] showed that there was a marked diminution in the creatinine excretion of hypophysectomised dogs. Relatively this effect comes on much sooner than the increased excretion of creatinine after castration, which indicates that the effect of castration is due primarily to the pituitary. The castration effect is retarded because it depends upon the gradual hypertrophy of the anterior lobe of the pituitary. The work of numerous investigators has established the fact that in many species castration leads to an increase in the weight of the anterior lobe [Moore and Price, 1932; Stein, 1933, who also give reviews of the literature]. Reese and McQueen-Williams [1932], have shown that the morphological changes in the anterior lobe of the male rat due to removal of the testes can be prevented by administration of extracts containing the active principle of the male gonads. The results of parabiotic experiments show that the hypertrophy is not due to defective utilisation and storage of hormones but involves increased production and secretion. Brauer [1929] found that anterior lobe hypertrophy after gonadectomy also occurs in rabbits although there seems to be some doubt whether females show any appreciable hypertrophy [Wolfe, 1932].

The depressing effect of the gonad secretions on the anterior lobe of the pituitary, only incompletely shown by the normal animal, is unmasked when the gonads are removed, and the effect of the removal of the inhibitory influence becomes more and more apparent as the anterior lobe gradually hypertrophies. This process is reflected in a gradual increase in the elimination of creatinine in the urine.

These facts together with those reported above afford strong evidence of a direct endocrine relationship between the anterior lobe of the pituitary and creatinine metabolism. The persistent increase in creatinine excretion which occurs as the result of castration implies the continued new formation of creatine and its transformation to creatinine. A steady level is reached after several months. This may be partly explained by the fact that hypertrophy of the anterior lobe reaches a maximum. Evans and Simpson [1929] noted in rats that the gonad-stimulating capacity of the pituitary slowly increased up to the 8th post-operative week, after which there was little increase, and Schenk [1927]

found that the cell-type changes in rats occur progressively until a level state is reached some time between the 4th and 7th post-operative months. On injection of anterior lobe extracts into castrated animals no further increase in the excretion of creatinine was obtained, and this indicates that cessation of pituitary hypertrophy is not the sole limiting factor in the production of a maximum steady level of creatinine output. It would be premature at this stage to attempt any detailed interpretation of the facts. Relevant data in regard to the creatine content of the muscles of normal and castrated animals and the effect of repeated daily injections of anterior lobe extracts are lacking.

(*b*) Injection of creatine.

2–5 g. of creatine were injected subcutaneously into normal and castrated rabbits and the creatine excretions estimated in the urine. The animals used in this experiment were the same as those used in the injection experiments reported in the first part of this paper. In Tables V and VI the figures in brackets refer to the amounts of creatine excreted in mg. No creatine was excreted by the control animal or by the other animals on the days preceding the injection.

Table V. *Daily urinary creatine and creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
27. iii. 33	100	120	136	92
28. iii. 33	100	120	132	91
29. iii. 33	98	114	120	96
30. iii. 33	102	125	135	92
31. iii. 33	109 (291)	126 (474)	141 (659)	92
1. iv. 33	109 (21)	135 (65)	126 (74)	88
2. iv. 33	96 (10)	120 (21)	126 (25)	92
3. iv. 33	100	126 (24)	133 (12)	92
4. iv. 33	100	120 (17)	133 (5)	96
5. iv. 33	105	126	133 (17)	92
6. iv. 33	100	120	135	92
7. iv. 33	105	123	126	96

On March 30 Nos. 1, 2 and 3 each injected with 2 g. creatine.

Table VI. *Daily urinary creatine and creatinine, mg.*

Date	Normal (1)	Castrated (2)	Castrated (3)	Normal (4)
2. v. 33	100	126	133	96
3. v. 33	100 (1144)	127 (1739)	120 (1746)	100 (1300)
4. v. 33	100 (71)	120 (413)	126 (310)	100 (108)
5. v. 33	92 (31)	120 (40)	130 (41)	105 (19)
6. v. 33	91	118 (18)	135 (23)	93 (6)
7. v. 33	92	130 (20)	126 (25)	96
8. v. 33	100	133	130	100
9. v. 33	90	120	124	95

On May 2 Nos. 1, 2 and 3 each injected with 5 g. creatine; No. 4 injected with 3·8 g.

The results of Tables V and VI are summarised below:

	Normal	Castrated	Castrated	Normal	Castrated	Castrated	Normal
Creatine injected (g.)	2	2	2	5	5	5	3·8
Total creatine excreted (mg.)	322	601	792	1245	2230	2145	1433
Creatine retained (mg.)	1678	1399	1208	3755	2770	2865	2367
Percentage excreted	16·1	30·0	40·0	25·0	44·6	42·9	37·7

It is evident that normal and castrated animals behave differently towards injected creatine. In all cases the normal animal excreted less creatine and retained more than the castrated, *i.e.*, the normal animals have a high and the castrated a low tolerance to exogenous creatine. The castrated animal excreted a larger proportion of the injected creatine and also took longer to do so. The excretion of creatinine was not affected.

These results are in essential agreement with those of Remen [1932]. He found that intravenous injection of 500 mg. creatine into normal adult men between the ages of 20 and 53 is followed by no greater excretion of creatine in urine than before injection. Men of 70–90 years, a eunuch 43 years old and one man of 68 castrated for carcinoma excreted most of the injected creatine. One case of hypophysial obesity, in which the normal excretion of creatine was high, reacted to injected creatine like normal adult men. The old men with failing sex function, the eunuch and the castrate thus showed a decreased tolerance typical of infants and children and, as shown above, of castrated rabbits. Remen concluded that the male sex glands have a regulatory influence on the metabolism of creatine. Lasch [1932] arrived at the same conclusion on the basis of similar experimental results. The evidence presented in the first part of this communication indicates that the difference between normal and castrated animals in regard to creatine-creatinine metabolism is due primarily to hyperactivity of the anterior lobe of the pituitary.

Summary.

1. Injection of antuitrin or of anterior lobe extracts of the pituitary leads to an increased excretion of urinary creatinine in normal rabbits but has no effect on the high creatinine output of castrated animals.
2. The evidence presented affords evidence of a direct endocrine relationship between the anterior lobe of the pituitary and creatinine metabolism.
3. Normal rabbits show a high, and castrated animals a low, tolerance to subcutaneous injections of large amounts of creatine.

The authors wish to thank Messrs Parke, Davis and Co., London, for a supply of antuitrin.

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CLXXXI. THE BIOLOGICAL RELATION BETWEEN CAROTENE AND VITAMIN A.

By JACK CECIL DRUMMOND and ROY JAMES MacWALTER.

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(*Received June 27th, 1933.*)

The experiments of Moore [1929], since amply confirmed by numerous workers, clearly established the appearance in the liver of vitamin A following the oral administration of carotene. It has been rather generally assumed that the conversion occurs in that organ, but direct evidence of a satisfactory character has not yet been advanced. Wolff *et al.* [1930] removed a portion of the liver from rabbits which had been fed on a diet devoid of both carotene and the vitamins. Following an injection of carotene into the circulation the vitamin A content of the residual liver tissue, determined by the increase in the antimony trichloride colour reaction, showed a considerable increase at the end of 3 days.

The first claim to have brought about the conversion *in vitro* was made by Olcott and McCann [1931]. They incubated buffered suspensions of liver and of an enzyme preparation, "carotenase," made from that tissue with colloidal solutions of carotene dissolved in ethyl laurate. A loss of colour was observed and after 24 hours the unsaponifiable fraction extract prepared from the digests showed on spectroscopic examination a discrete absorption band at 3280 Å. The changes were not observed when the "carotenase" had been inactivated by heating.

The disappearance of the colour of the carotene in such incubation experiments, is, as Woolf and Moore [1932] point out, of no particular significance, but serious attention must be given to what appears to be clear evidence of the formation of a product with selective absorption at 3280 Å. and to the statement that no such substance is formed when the liver extracts have been heated.

One confirmation of the claims of Olcott and McCann has been reported by Parienti and Ralli [1932]. They detected an increased response to the antimony trichloride reaction after incubating colloidal solutions of carotene with minced dog liver, buffered at p_H 7·4. They also state that "carotenase" is inactivated by heating. Other investigators have failed to confirm these observations and the position at the moment is not satisfactory. Von Euler and Klussmann [1932] claimed that the absorption at 3280 Å. of cow-liver extracts was increased threefold after incubation with colloidal carotene, but in another communication [v. Euler, 1932] it is stated that "die Versuche von Klussmann mit kolloidalem Carotin zum Leberbrei in Boratpuffer konnten die Ergebnisse von Olcott und McCann nicht bestätigen."

In this laboratory Ahmad [1931] and Rea and Drummond [1932] failed to detect the production of a substance resembling vitamin A from carotene either by incubating with liver preparations *in vitro* or by perfusing into the portal circulation of surviving cats.

The failure of the latter technique was rather surprising and suggested that the tissues of a typical carnivore might not be well adapted for efficiently performing the conversion; it must be remembered that evidence is accumulating to show that the carotene molecule may be degraded or modified in a variety of ways in the animal organism.

In the work to be described the investigation has been extended to cover a typical example of the herbivores. In general the procedure has been similar to that already described.

Experimental.

Preliminary experiments to determine the distribution of vitamin A throughout the liver were made, and it soon became apparent that rather large variations were to be expected.

The liver samples were dried by intimate mixing with anhydrous sodium sulphate, and the fatty material was rapidly extracted in a continuous extractor by hot chloroform. The extracts were examined spectroscopically for pigments and for vitamin A. For this work a new form of photometer recently placed on the market by Adam Hilger Ltd., was used—the Notched Echelon Wedge Cell. This instrument possesses the advantage of enabling the estimations to be made with great rapidity; a matter of great importance when dealing with such labile substances as carotene and vitamin A which are easily decomposed photochemically.

The experiments on the distribution of vitamin A in the liver of rabbits were made on two different groups of animals. The first had been maintained for some 8 weeks on a diet of oats and bran in order to lower their tissue reserves of that substance. Those in the second group had received the usual stock laboratory rabbit diet containing ample supplies of green leaves.

In Tables I and II the figures referring to vitamin A concentration are expressed as the extinction coefficient ($\log_{10} \frac{\text{light transmitted}}{\text{light incident}}$ referred to 1 cm. of solution) at 3280 Å., at such a concentration that 1 g. of liver is contained in 1 cc. of the chloroform extract.

Table I.

Number	I	II	III	IV	V	VI	VII	VIII	IX
Weight, kg.	1·2	1·8	1·93	—	—	1·4	1·7	—	—
Diet	−A	−A	−A	—	—	Normal		—	—
Left anterior lobe:									
Weight, g.	21·9	3·7	7·6	6·4	43	8·65	21·55	26·4	25·0
Vitamin concentration	2·65	4·5	3·55	8·2	2·5	6·9	2·95	17·1	3·7
Fat concentration	—	—	—	—	0·0173	0·0337	0·0175	0·0264	0·0182
Caudate lobe:									
Weight, g.	3·1	—	10·9	11·2	30	6·6	17·3	25·1	27
Vitamin concentration	2·0	—	—	12·2	3·4	6·8	3·6	12·20	6·1
Fat concentration	—	—	—	—	0·0104	0·0279	0·0200	0·0344	0·0157
Left posterior lobe:									
Weight, g.	12·8	11·35	9·7	16·90	56·5	9·5	23·60	26·3	23·0
Vitamin concentration	2·2	4·75	3·55	7·1	2·1	7·6	3·05	11·8	5·9
Fat concentration	—	—	—	—	0·0069	0·025	0·0137	0·0351	0·0201
Right lobe:									
Weight, g.	9·6	10·35	12·8	22·6	52·5	9·9	20·25	28·8	26·0
Vitamin concentration	—	5·0	3·0	9·2	3·8	7·2	3·65	17·2	6·6
Fat concentration	—	—	—	—	0·0137	0·0265	0·0172	0·0324	0·0164
Maximum deviation from mean %	16	6	10	31	29	4·2	12	19	33·7

The results show that the distribution of vitamin A in these rabbit livers is far from being uniform. The differences are greater in the normally fed animals than in those which had been deprived of vitamin A in their diet. In most cases they are definitely greater than the experimental error, which we estimate at 5 %. Curiously, no sharp correlation was found between the distribution of fatty material extracted by chloroform and the vitamin, as might reasonably have been expected.

The injection experiments were made by the following procedure. A portion of one lobe of the liver was removed aseptically under ether anaesthesia and a sterile colloidal suspension of carotene in 5 % glucose solution was slowly injected directly into the portal vein. The carotene suspension was prepared according to the directions of Fodor and Schoenfeld [1931]. After the injection the incision was closed and the animal kept for a suitable period before removing the remainder of the liver for examination. These periods extended from 24 hours to 20 days and in most cases care was taken to examine separately the residual portion of the lobe from which the original sample had been removed.

Some of the results are summarised in Table II.

Table II.

Rabbit No.	I	II	III	IV	V
Weight, kg.	1·8	1·57	1·05	2·5	1·97
Duration of experiment, days	1	5	5	8	20
Liver, excised portion:					
Weight, g.	3·30	4·9	2·5	3·7	2·6
Vitamin content	4·8	11·0	2·02	12·0	3·2
Basal portion of same lobe:					
Weight, g.	—	—	4·1	7·64	9·10
Vitamin content	—	—	1·7	15·0	8·7
Residual portion:					
Weight, g.	45·0	65·0	49·8	40·30	54·3
Vitamin content	4·4	9·3	2·0	14·0	6·0
% Increase on mean	−8	−15·4	−8·4	+20·8	+130
Carotene:					
Injected, mg.	0·69	0·70	0·6	1·2	1·12
Recovered, mg.	0·35	0·52	0·42	0·9	0·80

These results provide no evidence of a definite increase in vitamin A in the liver until 8 days have elapsed after the injection of the carotene. Observation showed that the injected pigment is rapidly taken up by the liver after introduction into the circulation. This was also noted by van den Bergh *et al.* [1920] who found that carotene injected in the form of a colloidal suspension into the jugular vein of rabbits was soon removed from the blood and to a large extent was held by the liver.

Our experiments show that some part of the carotene is broken down or disappears from the liver, although the loss is not nearly so large as was observed in our earlier experiments with cats. We cannot exclude the possibility that formation of vitamin A had occurred in all our experiments, for it is conceivable that the depleted organism might immediately utilise the material for resumption of growth or repair of structure. If such were true a measurable increase in the amount present in the liver would, presumably, only be detected after the requirements of the body had been satisfied and storage had become possible.

The resumption of growth shown by the rabbits kept for the longer periods after operation, in which storage of new vitamin A was detected, rather suggests that this may be true (Fig. 1).

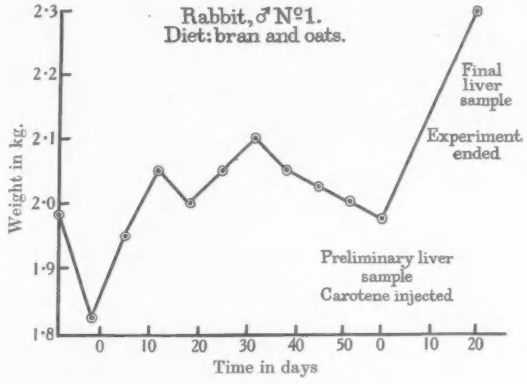

Fig. 1.

This work has been supplemented by a new series of experiments made to try and reproduce the conditions under which Olcott and McCann observed conversion of carotene into vitamin A *in vitro*. In every case the results were negative. Another method of attack was therefore devised, which it was thought would lead to success. The liver of a rabbit which had received an injection of colloidal carotene into the portal vein was removed after an interval sufficient to ensure that the pigment had been taken up in a normal manner from the circulation by the hepatic cells. Sterile preparations of the minced tissue suspended in phosphate buffer at p_H 7·3 were incubated at 37° for different periods. Afterwards the materials were extracted by chloroform and the extracts examined spectroscopically. Table III gives the results of four such experiments, but before stating the conclusion to which they lead us it is necessary to explain

Table III.

Number	Weight of liver, g.	Duration hours	Absorption at 3280 Å.	Absorption at 4800 Å.	Correction	Corrected absorption at 3280 Å.
I	2·21	0	5·5	—	—	—
	4·42	0	4·3	—	—	—
	2·17	48	5·75	—	—	—
	2·32	48	5·75	—	—	—
II	3·68	0	4·0	2·7	0·65	3·35
	3·77	24	3·95	2·7	0·9	3·05
	4·43	65	2·9	1·9	1·5	1·4
III	4·28	0	5·1	5·0	0·83	4·27
	0·72	0	5·1	5·0	0·83	4·27
	5·59	48	5·1	5·0	3·6	1·5
	4·16	120	5·1	2·0	2·0	3·1
	1·18	168	6·2	2·9	4	2·2
IV	4·80	0	17·7	1·8	0·3	17·4
	5·18	48	8·2	0·645	0·43	7·77
	7·14	120	7·0	0·645	0·645	6·38
	6·62	168	18·6	0·883	1·26	17·34
	6·53	168	18·6	0·645	0·92	17·68

the correction which it has been found necessary to apply in estimating the absorption in the region 3280 Å. We have confirmed the observations of McNicholas [1931], who found that solutions of carotene in ether and alcohol mixtures exposed to diffuse sunlight undergo oxidative breakdown in two stages. The initial changes are characterised by the appearance of an absorption band in the region of 3200 Å. with a corresponding decrease in the band at 2730 Å. The later stage is marked by a decrease in the colour of the solution and a widening of the ultra-violet bands which finally lose their entities.

A colloidal solution of carotene, such as we employed for our intravenous injections, when incubated at 37° in contact with air shows such changes. In Fig. 2 is represented the relation between the intensity of absorption at 4800 Å. and that at 3280 Å. as it changes during a period of 7 days; by 14 days the colour of the carotene had completely disappeared.

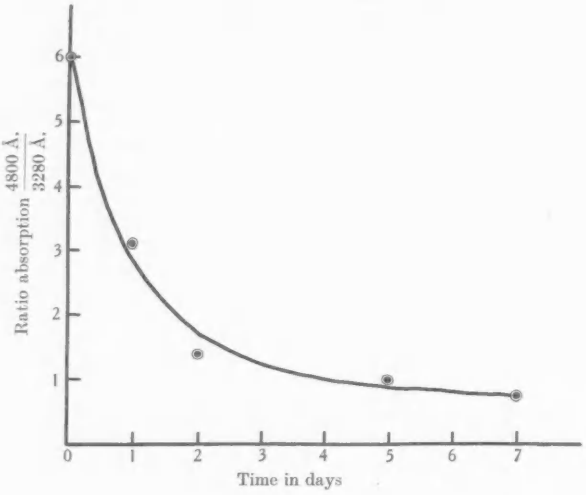

Fig. 2.

It is necessary, therefore, to apply a correction based on such a curve as that presented in Fig. 2 for all estimations of absorption at 3280 Å. made on preparations of carotene incubated in the presence of air. When such corrections are applied our results indicate that the incubated liver containing absorbed carotene shows at first a decreased absorption at 3280 Å. followed by a gradual increase. But a similar increase is shown by liver preparations to which carotene has not been added.

It is, however, impossible to apply a similar correction for the estimations made on the perfusion experiments described earlier in the paper because our observations show that a considerable proportion of the carotene may remain unchanged in the liver for periods as long as 20 days. In this organ the changes appear to be less rapid than in the solutions exposed to air.

SUMMARY.

After injecting carotene into the portal circulation of rabbits no increase in the vitamin A in the liver was observed, using a spectrophotometer method, until 8 days after the experiment. There is of course the possibility that formation of vitamin occurs within much shorter periods, but either that it is utilised and converted into another substance or that the amounts are too small to be detected by our technique. Further attempts to obtain conversion of carotene into vitamin A *in vitro* by incubation of liver tissues have met with no success.

In some of these experiments the conditions were made more "physiological" by permitting the liver cells to take up the pigments from the circulating blood before the minced preparation was incubated.

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CLXXXII. AN INVESTIGATION OF THE CAUSE OF RENAL HYPERTROPHY IN RATS FED ON A HIGH PROTEIN DIET.

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The problem of the enlargement of the kidneys of rats fed on diets rich in protein has, in recent years, been receiving considerable attention notably by Addis and the Mackays [1926, 1, 2] who have shown that the hypertrophy is proportional to the protein consumption and have elaborated a formula which expresses this relationship in terms of protein consumed and kidney weight per unit of surface area. The cause of this enlargement, however, still remains obscure. The excretion of urea naturally suggests itself as a causal factor. The feeding of urea, however, in amounts equivalent to what would be excreted on a high protein diet has been shown to produce a much smaller effect on the kidneys [Osborne *et al.*, 1926; Mackay, Mackay and Addis, 1931]. The effect of feeding proteins of different origin has however scarcely been investigated. Osborne *et al.* [1926] found that caseinogen, gliadin and beef residues all produced an enlargement of the kidneys but no actual numerical data on the basis worked out by the Mackays and Addis [1927; 1928] were elaborated. The object of the following experiments was an attempt to find out the cause of this hypertrophy.

Two possibilities suggest themselves. Do the kidneys in the rat form a depot for reserve protein, or is the hypertrophy a response to an increase in physiological function? It is possible in this connection that the kidneys are associated in some way with the intermediary metabolism of protein quite apart from the mere excretion of nitrogenous end-products.

Experimental.

A series of experiments was carried out to compare the following proteins and protein derivatives, and in addition the effect of an acid and alkaline diet—caseinogen, liver, gelatin, wheat gluten, glycine, glutamic acid, urea, NaH_2PO_4 and $NaHCO_3$. The experimental procedure adopted in these experiments was practically the same as that employed by the Mackays [1927]. Standard albino rats 30 days old and obtained from the same source as far as each particular series of experiments is concerned were employed throughout. They were kept in cages open to the front with not more than 6 rats to a cage. The sexes were kept apart. The room in which they were kept was well lit and open to the south and was maintained fairly constant in temperature day and night. Water and food were allowed *ad lib.*, and the food consumption was measured each day. The rats were weighed about every third day. The duration of the experiments varied from 30 to 39 days in the different series. At the end of the experimental

period the rats were killed by chloroform and weighed. The abdomen was then opened, the kidneys removed, decapsulated, split in half, dried on filter-paper and weighed in a glass-stoppered bottle. The two kidneys were weighed together and the average taken as the weight of one. The surface area was calculated by Meeh's formula $S = BW^{\frac{2}{3}} \times K$. The constant $K = 11{\cdot}36$ was that employed by the Mackays [1927] and found to be applicable to the rat by Carman and Mitchell [1926]. From the experimental data the following calculations have been made for each series. The average increase in body weight per rat daily; the average kidney weight per 100 cm.^{2} body surface. The average daily protein consumption per 100 cm.^{2}; the average increase in body weight per g. of protein consumed.

The first series of experiments was carried out with liver, gelatin and caseinogen. The object of testing liver was partly because it offers a protein differing considerably from caseinogen and partly because it is rich in extractives and nucleoproteins. Gelatin was chosen as a test protein because it has a low biological value and hence might be considered inadequate to build up any reserve tissue in the kidney. A further point of note is that gelatin is poor in tryptophan, cystine and tyrosine and according to certain workers [Newburgh and Marsh, 1925] these amino-acids are a contributing cause of the renal degeneration found in rats fed over a long period on a high protein diet. The diets employed are given in Table I and the results in Table II.

Table I. *Diets employed.*

(Figures represent parts per 100.)

Series	Dried liver	Casei-nogen	Gela-tin	Glu-ten	Gly-cine	Glu-tamic acid	Dex-trin	Lard	Urea	Salt mix-ture	Cod-liver oil	Dried yeast	Agar	$NaHCO_3$	NaH_2PO_4	Total protein %	Total Cals.
A	80	—	—	—	—	—	—	—	—	3	6	9	2	—	—	50·5	434
B	—	16	37	—	—	—	15	8	—	3	10	9	2	—	—	50·6	436
C	—	57	—	—	—	—	9	10	—	3	10	9	2	—	—	50·0	436
D	—	20	32	—	—	—	13	5	—	3	10	9	2	—	6	48·8	396
E	—	20	32	—	—	—	13	5	—	3	10	9	2	6	—	48·8	396
F	—	57	—	—	—	—	8	5	—	3	10	9	2	—	6	48·8	402
G	—	20	—	—	—	—	51	5	—	3	10	9	2	—	—	20·25	428
H	—	20	32	—	—	—	17	7	—	3	10	9	2	—	—	48·9	431
I	—	57	—	—	—	—	10	10	—	3	10	9	2	—	—	48·9	429
J	—	20	—	—	—	—	51	5	—	3	10	9	2	—	—	20·6	428
K	—	57	—	—	—	—	9	10	—	3	10	9	2	—	—	48·5	429
L	—	20	—	—	—	—	34·2	12	9·8	3	10	9	2	—	—	48·8*	426
M	—	20	32	—	—	—	17	7	—	3	10	9	2	—	—	48·5	431
N	—	20	—	—	—	—	56	—	—	3	10	9	2	—	—	20·2	402
O	—	20	—	26	—	—	30	—	—	3	10	9	2	—	—	42·7	402
P	—	20	—	—	10	—	42	4	—	3	10	9	2	—	—	31·4	403
Q	—	20	—	—	—	25	27	4	—	3	10	9	2	—	—	34·1	402

* Urea-N calculated as protein.

It will be noted that there is no difference in the kidney weights of groups A and C, *i.e.* those fed with liver and caseinogen respectively. The rats on the gelatin diet (B) showed however a considerable hypertrophy in comparison with groups A and C in spite of the fact that they actually consumed less protein. In order to ascertain if the differences between the groups are significant the standard deviation σ was calculated for each group and the probable error of the difference of the means calculated by the formula $0{\cdot}6745\sqrt{\frac{\sigma_1}{n_1}+\frac{\sigma_2}{n_2}}$ where n_1 and n_2 are the number of observations in each group and σ_1 and σ_2 the corresponding standard deviations. Where the ratio of the difference of the means to the probable error of the difference of the means is 3 or more the result is considered significant. The following are the data for series I.

A	σ	B	σ	C	σ
Males	0·03507	Males	0·00319	Males	0·02097
Females	0·01552	Females	0·0900	Females	0·02097

A and B males:	Difference of means	0·066
	Probable error of difference of means	0·0113
	Ratio	5·84 : 1
A and B females:	Difference of means	0·103
	Probable error of difference of means	0·025
	Ratio	4·12 : 1
B and C males:	Difference of means	0·084
	Probable error of difference of means	0·0082
	Ratio	10·24 : 1
B and C females:	Difference of means	0·099
	Probable error of difference of means	0·025
	Ratio	3·96 : 1

It will be seen that the difference is significant when the group fed with gelatin is compared with either of the groups fed with caseinogen or liver. It is possible that the increase in kidney weight of group B (gelatin) is due to the

Table II.

			Av. increase in body weight per rat per day, g.	Av. kidney weight per 100 cm.^{2} g.	Av. protein consumption per day per 100 cm.^{2} g.	Av. increase in body weight per rat per g. protein consumed
Series 1 31 days exp.	A	7 ♂	3·07	0·285	1·64	0·57
		7 ♀	2·10	0·239	1·57	0·52
	B	9 ♂	2·13	0·351	1·33	0·59
		6 ♀	1·76	0·342	1·58	0·46
	C	5 ♂	3·70	0·267	1·38	0·75
		12 ♀	2·03	0·243	1·39	0·51
Series 2 32 days exp.	D	6 ♂	1·12	0·403	1·50	0·32
		6 ♀	0·96	0·363	1·33	0·30
	E	6 ♂	2·0	0·395	1·43	0·46
		6 ♀	1·09	0·338	1·27	0·33
	F	6 ♂	3·05	0·324	1·41	0·62
		6 ♀	2·02	0·348	1·33	0·53
Series 3 39 days exp.	G	5 ♂	2·96	0·265	0·65	1·14
		6 ♀	1·67	0·227	0·67	0·81
	H	6 ♂	1·95	0·362	1·39	0·47
		6 ♀	1·34	0·340	1·39	0·38
	I	6 ♂	2·90	0·265	1·28	0·63
		6 ♀	1·60	0·237	1·38	0·40
Series 4 32 days exp.	J	6 ♂	2·01	0·218	0·67	1·07
		6 ♀	1·39	0·196	0·70	0·80
	K	6 ♂	1·73	0·221	1·14	0·59
		6 ♀	1·62	0·246	1·52	0·45
	L	6 ♂	1·89	0·236	1·44	0·52
		6 ♀	1·55	0·217	1·44	0·43
	M	6 ♂	1·63	0·369	1·49	0·47
		6 ♀	1·20	0·336	1·69	0·32
Series 5 32 days exp.	N	5 ♂	1·91	0·212	0·65	1·26
		5 ♀	1·57	0·199	0·64	1·16
	O	5 ♂	1·79	0·304	1·31	0·59
		5 ♀	1·31	0·270	1·31	0·47
	P	3 ♂	0·65	0·278	1·10	0·59
	Q	4 ♀	1·17	0·244	1·12	0·52

fact that although this group consumed less protein than groups A and C they actually catabolised more nitrogen. This assumption might be expected *a priori* since gelatin is almost incapable of promoting growth. This explanation would assume however that the hypertrophy is observed only when the protein is actually catabolised. In point of fact, as is seen from Table II, the increase in body weight per g. of protein consumed is approximately the same in groups A and B. It cannot however be inferred that the material added to the body in each group had the same nitrogen content.

In series 2 an attempt was made to repeat the work of Addis, Mackay and Mackay [1926, 1, 2] on the influence of adding acid or alkali to the diet. Nash and Benedict [1921] have shown that the kidney, in the dog at least, is the main if not the only organ concerned in the formation of ammonia and it was thought possible that the kidney hypertrophy might be secondary to an increased activity of this function following a high protein diet. The salts employed in the experiments were sodium dihydrogen phosphate and sodium bicarbonate. In experiments D and E (Table I, series 2) the diets were identical except that D was acid and E alkaline. The additional protein in D and E was gelatin. Mackay, Mackay and Addis [1926] have shown that phosphates themselves cause a considerable increase in kidney weight, and hence in groups D and E it would be possible to see if the hypertrophy of the kidneys in group D with gelatin and acid phosphate would be greater than in group E with gelatin and alkali. In short would there be a summation of those two factors in bringing about a hypertrophy? The experimental data are given in Table II, series 2.

It will be noted that there is scarcely any difference between groups D and E fed with gelatin and acid and alkali respectively. The difference in the kidney weights for the males is only 0·008 g. and for the females 0·025 g. per 100 cm.^{2} body surface. Such differences are not significant. It thus appears that there is no summation of the effects of gelatin and phosphates when fed together in a diet. In group F fed with caseinogen *plus* acid phosphate the effect of the salt is quite marked. This is seen most clearly when groups C and F of series 1 and 2 are compared. The following are the statistical data.

D	σ	E	σ	F	σ
Males	0·03014	Males	0·0250	Males	0·0442
Females	0·07200	Females	0·0375	Females	0·0820

Series 2, D and F males:	Difference of means	0·079
	Probable error of difference of means	0·0147
	Ratio	5·37 : 1
Series 2, E and F males:	Difference of means	0·071
	Probable error of difference of means	0·139
	Ratio	5·07 : 1
Series 1 and 2, C and F males:	Difference of means	0·057
	Probable error of difference of means	0·01371
	Ratio	4·15 : 1
Series 1 and 2, C and F females:	Difference of means	0·105
	Probable error of difference of means	0·0214
	Ratio	4·90 : 1

Taking series 2 alone to begin with it will be noted that the difference between group D (gelatin and acid phosphate) and group F (caseinogen and acid phosphate) is significant in respect of the males. The kidney weights of the females of those two groups are however approximately the same. This contrast in the effect of caseinogen in series 2 as compared with series 1 must be attributed to the phosphates as the statistical data show when groups C and F of series 1 and 2

are compared above. The difference is significant for both sexes. The evidence from these experiments does not appear to favour the hypothesis that acidity or alkalinity of the diet, and hence ammonia formation by the kidney, is a factor in the renal hypertrophy. In series 3 an attempt was made to test the relative effect of superimposing caseinogen on the one hand and gelatin on the other on a diet low in caseinogen. The diets employed and the experimental data are given in Tables I and II, series 3 (G, H, I). Contrary to the findings of the Mackays [1927] there is no increase in the kidney weight when the protein of the diet is increased by the addition of extra caseinogen (groups G and I). In group H however where the increase in the protein of the diet is in the form of gelatin a hypertrophy of the kidneys is to be noted. The following are the statistical data.

G	σ	H	σ	I	σ
Males	0·03301	Males	0·02696	Males	0·02720
Females	0·03684	Females	0·03390	Females	0·02260

Series 3, G and H males:	Difference of means	0·097
	Probable error of difference of means	0·0124
	Ratio	7·82 : 1
G and H females:	Difference of means	0·113
	Probable error of difference of means	0·0137
	Ratio	8·25 : 1
H and I males:	Difference of means	0·097
	Probable error of difference of means	0·0101
	Ratio	9·63 : 1
H and I females:	Difference of means	0·103
	Probable error of difference of means	0·112
	Ratio	9·19 : 1
G and I males:	Difference of means	Nil
G and I females:	Difference of means	0·010

The increase in weight between groups G and H and H and I is very marked and merits special attention when comparing groups H and I which had practically the same percentage of protein in the diet and consumed almost the same quantity of protein per 100 cm.^{2} body surface. Owing to the fact that additional caseinogen failed to cause a hypertrophy in the above series it was decided to repeat the experiments and in addition to compare the effect of urea and gelatin respectively with each other and with caseinogen. The diets employed and the experimental data are given in Tables I and II, series 4. Below are recorded the statistical results (J, K, L, and M).

The results in general confirm those of series 3. The increase in kidney weight on the high protein diet (K) as compared with the low (J) is significant with the female but not with the male group. The increase in kidney weight with the group fed with urea (L) as compared with the low protein (caseinogen) group (J) is significant but the differences are much less than those between groups J and M where the extra nitrogen is consumed in the form of gelatin. The difference is very marked in the case of groups L and M which consumed approximately the same amount of nitrogen—group L largely as urea and group M as gelatin. Assuming that the gelatin was all catabolised and excreted as urea, this experiment would confirm the fact that the excretion of urea is not the causal factor in the kidney hypertrophy. From the experiments in series 3 and 4 an estimate can be made of the increase in kidney weight per 100 cm.^{2} body surface per

J	σ	K	σ	L	σ	M	σ
Males	0·0122	Males	0·0232	Males	0·0104	Males	0·0264
Females	0·0187	Females	0·0176	Females	0·0150	Females	0·0264

Series 4, J and M males:	Difference of means	0·151
	Probable error of difference of means	0·008
	Ratio	18·8 : 1
J and M females:	Difference of means	0·140
	Probable error of difference of means	0·008
	Ratio	17·5 : 1
J and K males:	Difference of means	0·003
	Probable error of difference of means	0·0072
	Ratio	0·041 : 1
J and K females:	Difference of means	0·050
	Probable error of difference of means	0·0067
	Ratio	7·64 : 1
J and L males:	Difference of means	0·018
	Probable error of difference of means	0·0044
	Ratio	4·09 : 1
J and L females:	Difference of means	0·021
	Probable error of difference of means	0·0062
	Ratio	3·38 : 1
L and M males:	Difference of means	0·133
	Probable error of difference of means	0·0078
	Ratio	17·02 : 1
L and M females:	Difference of means	0·119
	Probable error of difference of means	0·009
	Ratio	13·22 : 1

additional g. of protein consumed. In series 3 this has been calculated where the increase in protein consumed was derived from gelatin. The following are the values found.

Series 3, males	0·131 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}
females	0·156 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}
Series 4, males	0·184 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}
females	0·141 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

These figures agree fairly closely with those obtained by the Mackays [1927] where the additional dietary protein was in the form of caseinogen.

In series 5 a cereal protein was tested and an attempt was made to find out which particular amino-acid or acids, if any, were responsible for the hypertrophy observed when feeding the whole protein. Wheat gluten, glycine and glutamic acid were selected as examples of cereal protein and amino-acids, glycine being chosen because gelatin contains a high percentage of this particular amino-acid and glutamic acid because of its high concentration in gluten. In the experiment with glycine 4 male rats were put on a diet containing 20 % of this substance; they tended to lose weight however and finally one died. The percentage of glycine was then reduced to 10 %. The animals in group Q however consumed a diet containing 26 % of glutamic acid quite readily. The diets and experimental data are given in Tables I and II (N, O, P and Q). It will be noted that both gluten and the amino-acids caused a hypertrophy of the kidneys. The following are the statistical data.

N	σ	O	σ	P	σ	Q	σ
Males	0·0275	Males	0·0356	Males	0·328		—
Females	0·0250	Females	0·0317		—	Females	0·0091

Series 5, N and O males:	Difference of means	0·094
	Probable error of difference of means	0·0135
	Ratio	6·96 : 1
N and O females:	Difference of means	0·070
	Probable error of difference of means	0·0122
	Ratio	5·37 : 1
N and P males:	Difference of means	0·066
	Probable error of difference of means	0·0152
	Ratio	4·34 : 1
N and Q females:	Difference of means	0·45
	Probable error of difference of means	0·0091
	Ratio	4·51 : 1

The differences in the weights of the kidneys per 100 cm.^{2} per additional g. of protein (calculated from nitrogen consumed) consumed per 100 cm.^{2} body surface have been calculated for the different groups, *e.g.* caseinogen-gluten where the additional protein refers to the last-mentioned.

N and O males (caseinogen-gluten) 0·139 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

N and O females (caseinogen-gluten) 0·105 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

N and P males (caseinogen-glycine) 0·146 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

N and Q females (caseinogen-glutamic acid) 0·093 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

O and P males ((caseinogen glycine)-(caseinogen gluten)) 0·123 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

O and Q females ((caseinogen glutamic acid)-(caseinogen gluten)) 0·136 g. increase in kidney weight per 100 cm.^{2} per extra g. protein consumed per 100 cm.^{2}

If comparisons are drawn between animals of like sex it will be seen that the increase in kidney weight is proportional to the additional protein, *i.e.* N consumed. The addition of gluten to the diet causes an increase in the weight of the male kidneys of 0·139 g. per 100 cm.^{2} body surface per additional g. of protein consumed. Comparing the females (N and O) on caseinogen and caseinogen *plus* gluten respectively with those on caseinogen and caseinogen *plus* glutamic acid respectively (N and Q) the differences are 0·105 g. and 0·093 g. per unit of surface per additional g. of protein consumed. Taking into consideration the nature of the experiment these results are fairly close, a difference of only 0·012 g. being observed between the groups. A similar comparison between the males of groups O and P (gluten and glycine respectively) against N as a standard shows a difference of only 0·007 g. between the groups. These results indicate that it is unlikely that any particular amino-acid is the factor concerned in the hypertrophy. It should be pointed out however that these differences are smaller than those obtained when gelatin is fed. In series 3 the figures giving the increase in kidney weight with gelatin as the protein superimposed are males 0·131 g. and females 0·141 g. per unit of surface area per additional g. of protein consumed. The corresponding figures of series 4 are males 0·184 g. and females 0·141 g. The averages of the two series—males 0·157;

females 0·148 g.—however, are approximately the same as those obtained by the Mackays [1927] where caseinogen at different levels was the protein fed. It will be recalled that in our experiments additional caseinogen caused a relatively small hypertrophy of the kidneys and in some cases none at all.

At this stage of the investigation however it cannot be said what is the cause of the hypertrophy. That it is a real increase in tissue substance and not a hydraemia has been shown in a recent paper by Mackay [1933], who found that the moisture content for all kidneys fell between 74 and 76 %. In confirmation of his findings the average water contents of the kidneys in series 5 exhibit the same consistency as shown below.

	H_2O %
Group N, males	75·1
females	73·7
Group O, males	76·5
females	76·4
Group P, males	76·0
Group Q, females	74·9

From the general trend of evidence however in the recent paper by Mackay [1933], which has just come to our notice, and from the results given in this paper, it appears that the cause of the hypertrophy is associated with some change common to all the amino-acids. Ammonia formation and deamination naturally suggest themselves. The first possibility appears unlikely in view of the experiments recorded here and by Addis and the Mackays [1926, 1, 2]. The question as to whether the kidney is capable of deaminating merits special attention in view of the work of Bollmann, Mann and Magath [1926], who have shown that, as far as the dog is concerned, the liver is the main if not the only organ capable of deaminating. Recent work by Holmes and Watchorn [1927] shows that the growing embryonic rat kidney produces ammonia and urea *in vitro*. Krebs [1932] has further shown that rat kidney *in vitro* is more active than rat liver in deamination and ammonia formation. Such results suggest strongly that, at least in the rat, the kidney may be concerned in deamination. In support of this hypothesis is the fact that the hypertrophy tends to be larger or more readily produced by the addition of gelatin than of caseinogen to the diet. It can be more or less assumed that ingested gelatin will be almost entirely catabolised while some of the caseinogen may readily be used for anabolic purposes. Experiments on the actual nitrogen excretion on the two diets would give information on this point. The application of these results to the treatment of renal disease can hardly be ventured as yet. Some of the kidneys of each group of series 3 were examined histologically and no abnormality was found as had already been noted by Osborne *et al.* [1926]. Newburgh and Curtis, however [1928], find definite signs of degeneration in the kidneys of rats fed over long periods on diets containing a high percentage of protein.

Summary.

1. The increase in kidney weight of rats fed with a number of proteins and protein derivatives has been investigated.

2. Gelatin tends to produce a more marked increase in kidney weight than either caseinogen or liver.

3. Glycine, glutamic acid and gluten all produce an increase which is more or less proportional to the additional nitrogen consumed.

4. It is considered likely that the hypertrophy is associated with some stage in the intermediary metabolism of protein—probably deamination by the kidney.

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CLXXXIII. THE OXIDASE AND DEHYDROGENASE SYSTEMS OF THE CRYSTALLINE STYLE OF MOLLUSCA.

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THE occurrence in the crystalline style of *Saxidomus giganteus*, *Paphia staminea* and *Mya arenaria* of substances having the general properties of enzymes and capable of oxidising some of the reagents commonly employed for the detection of oxidases was recorded in a previous paper [Berkeley, 1923]. Similar substances have since been found in the style of *Pecten maximus*, *Mytilus edulis*, *Ostrea edulis* [Yonge, 1925; 1926, 1]. *Ensis siliqua* [Yonge, 1925; Graham, 1931], *Schizotheurus nuttalli*, *Ostrea lurida*, *Ostrea virginica*, *Ostrea gigas*, *Bankia setacea* and *Cardium corbis* [Berkeley, 1933].

The observations that the oxidation of guaiacum could be brought about by an extract of the style of *Saxidomus giganteus* in the absence of free oxygen and that the style disappeared from animals kept in boiled water in sealed vessels and was regenerated on access of air led me to suggest that the style might play a part in the anaerobic respiration of lamellibranchs first recorded by Collip [1920]. Doubt has been thrown on the suggestion that there is any essential connection between the disappearance of the style and anaerobic respiration by Nelson [1925] and by Yonge [1925; 1926, 2]. Nelson contends that anaerobic conditions slow down the movement of the cilia of the style-sac and the activity of the style-secreting cells; the material of the style dissolved in the stomach is not replaced and the style therefore disappears quite apart from any possible part it might play in supplying oxygen. He also points out that no relation can be found between the sizes of the styles of molluscs living normally under very well and very poorly aerated conditions respectively. Yonge brings forward the same arguments and also shows that the disappearance of the style depends upon the relation between its p_H and that of the gastric fluid. The style has invariably the lower p_H, so that the portion of it which is exposed to the action of the stomach contents is normally dissolved, and its maintenance depends entirely on its continuous renewal by the secreting cells.

Whilst there can be no doubt of the validity of these contentions it still remained possible that an oxidation process brought about by the oxidase set free by the dissolution of the style might lead to the production during anaerobiosis of some or all of the CO_2 on the production of which the evidence for anaerobic respiration rests. This possibility could not be disposed of until the actual physiological reaction brought about by the activity of the oxidase was determined. The present paper deals with an attempt to determine this reaction.

The autoxidisable substance of the oxidase system.

The style of *Saxidomus giganteus* has been used throughout the experiments to be described. It was stated in my previous paper [Berkeley, 1923] that active oxidase preparations were obtained when styles of this clam were dried rapidly

at air temperature *in vacuo*. Further experience has led to a modification of this finding. It is true that the dried material, when freshly prepared, yields active aqueous extracts, but it has been found that it loses the power of so doing after keeping for a few weeks, even in a tightly closed container. The loss of activity is due to the destruction of the autoxidisable component of the oxidase system, and not to that of the peroxidase, since it is restored on addition of hydrogen peroxide to the extracts. It has further been found that, in order to obtain active oxidase preparations from undried styles taken from freshly-dug clams, it is essential that the anterior ends of the styles, which impinge on the gastric shield, be left intact. If these are removed preparations made from the remaining portion of the styles have little[1] or no oxidase activity. They are rendered active by addition of either hydrogen peroxide or of an extract of the anterior end of the style.

This observation led to the conclusion that the autoxidisable component of the oxidase system was in some way connected with the anterior end of the style. In clams which have recently been feeding this end of the style always carries a mass of finely ground food material consisting mainly of bottom-living diatoms. It was suspected that these might be the source of the autoxidisable substance, and experiments were therefore made with aqueous extracts of similar diatoms. A mass of these organisms was scraped off the glass sides of an aquarium tank. The material weighed about 2 g. after draining and drying as completely as possible with filter-paper, and consisted almost entirely of *Navicula* spp. It was ground with a little distilled water until microscopic examination showed that most of the cells were ruptured; more water was added and grinding continued for a further period. The material was then saturated with toluene, the volume made up to 15 cc. and allowed to stand for an hour or so. It was then centrifuged until microscopic examination showed practically no solid particles in suspension. Half a gram of crystalline styles dried *in vacuo* some weeks previously was finely ground and allowed to stand in 15 cc. of distilled water saturated with toluene for 17 hours at air temperature. At the end of this time most of the material had dissolved. The solution was centrifuged and tested for oxidase activity, with and without addition of an equal volume of the diatom extract, by the indophenol reaction and by means of *p*-phenylenediamine, α-naphthol and pyrogallol. The diatom extract, without addition of the style extract, was tested simultaneously. The mixed extracts gave the most rapid and strongest reactions with each reagent. The style extract alone gave a slight indophenol reaction, but did not oxidise the other reagents. The diatom extract alone showed some slight activity towards pyrogallol, but was practically without action on the other reagents. The rather dark yellow-green colour of the diatom extract prevented the satisfactory use of guaiacum as a reagent for these tests owing to the difficulty of detecting the development of blue. A parallel series of tests was carried out, however, using an extract of plankton diatoms (obtained by towing in the open sea and consisting mainly of *Chaetocerus* spp.) made in the same manner. The reactions obtained were similar and, in this case, the guaiacum reaction could be applied since the diatom extract was only slightly coloured. It confirmed the other tests; the style extract alone being quite inactive, the diatom extract very slightly so, and the mixture giving a strong and rapid reaction.

[1] Removal of the anterior end of the styles eliminates the greater part, but not all, of the diatom food-material. Some inevitably remains both on and in the style. This probably accounts for the fact that extracts of styles which have had their anterior ends removed sometimes show slight oxidase activity.

It was evident that the autoxidisable component of the oxidase system of the style was contained in the diatoms adherent to it. A confirmation of this was obtained from clams which had been kept in the sea in a floating cage during a period of the year when the surface water contained little or no plankton. As a consequence the clams had been unable to feed and the anterior ends of their styles were quite free from diatoms. An extract of these styles was entirely without oxidase activity, but it was established by addition of either an extract of diatoms or of hydrogen peroxide.

The nature of the diatom constituent involved has yet to be determined. It does not seem to be as unstable as was to be anticipated from the rather rapid loss of oxidase activity by the dried styles, so long as the diatoms remain intact. Some of the bottom diatom material used in the previous experiments was dried *in vacuo* and kept for some weeks. It was then ground and extracted and a series of tests carried out with the extract to determine its power of inducing oxidase activity in an inactive style extract relatively to that of an extract of undried diatoms. The two diatom extracts were made as nearly as possible of the same concentration. The dry material led to only slightly less oxidising activity than the wet, indicating that little destruction of the autoxidisable substance had resulted from drying and storage. The destruction of the autoxidisable substance is presumably due to oxidation by atmospheric oxygen, and it is probable that this occurs more rapidly in the material occurring naturally on the style than in the intact diatoms owing to the fine state of division to which it has been reduced. Filtration of diatom extracts removes the autoxidisable substance almost entirely.

Since it was not always possible to obtain live clams as they were required, extracts of dried styles, which were inactive without addition of the autoxidisable substance, and of fresh diatoms, made as described above, have been used for a large part of this work. Moreover, this course had the advantages that solutions of greater oxidising activity could be made than were obtained by using fresh styles and that the parts played in their joint action by style extract and diatom extract respectively could be separately studied.

Selection of substrate.

Since all molluscs which have a crystalline style are herbivores [Yonge, 1930], and the style is mainly concerned in grinding food the organic portion of which consists largely of polysaccharides and submitting it to preliminary digestion, it was anticipated that the substance acted upon by the oxidase was a carbohydrate. It has long been known that the style contains an amylolytic enzyme, but there is no evidence that the style alone can carry the hydrolysis of starch to the formation of glucose, indeed evidence to the contrary has been brought forward by Yonge [1926, 1, 2], who showed that maltase is not present in the style of *Ostrea edulis*, though it occurs in the digestive diverticula. The production of glucose certainly occurs after the ground food mixed with style material leaves the stomach, and it is unlikely that any oxidative process would take place until this stage of hydrolysis had been reached. Accordingly it was decided to experiment in the first place with glucose as substrate.

Acid formation.

All attempts to detect the formation of an acid substance by the action of style extract on glucose failed. Freshly dug clams were used for these experiments and care was taken to retain the anterior portion, carrying the diatom

mass, in dissecting out the styles. These were dissolved in distilled water, the p_H of which had been adjusted at 7·0, saturated with toluene, using 1 style (weighing on average 0·2 g.) per 1 cc. of water. Solution was almost complete after standing for a period of 12 hours at air temperature, at the end of which the small amount of undissolved material was removed by centrifuging. Solutions thus prepared reacted strongly with oxidase reagents. Their p_H ranged from 6·2 to 6·0. The p_H of the style of *Saxidomus giganteus* is thus higher than that recorded for other lamellibranchs [Yonge, 1931]. Quantities varying from 1·25 to 5 % of pure glucose were added to tubes each containing 5 cc. of such style solutions and bromocresol purple as indicator. A set of tubes was allowed to stand at air temperature and another in a bath kept at 30° to 35°. No change of p_H could be observed in any of the tubes during periods up to 3 days. It seemed probable that the large amount of protein present in the style extracts exerted so strong a buffering action that an actual development of acid produced no corresponding alteration in p_H. It was found, however, that addition of one drop of a 0·01 % solution of formic acid to any one of the tubes produced a recognisable alteration in the colour of the indicator. It was concluded, therefore, that if the glucose were undergoing oxidation, the process did not result in the production of an acid.

Production of a substance giving the tests for glucosone.

Since glucosone occurs in addition to acids amongst the products of the mild oxidation of glucose an attempt was made to identify this substance as a reaction product of the style extract on glucose. For this purpose a test was necessary which would detect glucosone in small concentration in the presence of relatively large amounts of glucose. A solution containing glucosone was prepared from pure glucose by oxidation with copper acetate, following the method of Evans *et al.* [1928]. Glucosone was not isolated. The solution, after removal of copper, was used direct for the purpose of standardising the tests, and calculations of concentration were made on the assumption that oxidation to glucosone had occurred at the rate indicated by Evans *et al.* under the experimental conditions laid down by them.

A solution of 0·5 g. of *m*-nitrophenylhydrazine in 30 cc. 2*N* HCl was found a satisfactory reagent. Equal volumes of the reagent and of the solution under test are mixed and allowed to stand at 37°. A 0·03 % solution of glucosone produces a precipitate in half an hour. A 5 % solution of glucose is without action in that time. Dixon and Harrison [1932] used a 2 : 4-dinitrophenylhydrazine reagent made up in the same way for the detection of glucosone. The reagents seem to be of about equal delicacy.

It will be shown later that positive reactions were obtained with this test in application to solutions of crystalline style containing glucose, but, since it was not found possible to obtain sufficient of the reaction product to identify it by melting-point or other quantitative means, it was considered advisable to supplement the test by the following two others.

I. *Reduction to fructose and detection by the Seliwanoff test.* Glucosone on reduction by zinc dust and acetic acid yields fructose which can be detected in very dilute solution by means of the Seliwanoff test. Since glucose in relatively concentrated solution reacts to some extent it is necessary to remove most of it from the solutions to be tested. This can be accomplished by oxidation with bromine water, which leaves fructose practically unaltered. The procedure employed is as follows. 1 g. Zn dust and 0·5 cc. of glacial acetic acid are added to 5 cc. of the solution under test and the mixture is heated in a loosely-corked

test-tube standing in boiling water for 2 hours. After cooling the undissolved Zn is filtered off and 20 cc. of bromine water (saturated at air temperature) are added. The mixture is allowed to stand at air temperature in a closed vessel for at least 4 days, after which it is evaporated on a water-bath to 5 cc. An equal volume of 25 % HCl is added to this and a trace of resorcinol and the solution is heated in boiling water for a minute and quickly cooled. The presence of 0·02 % of glucosone produces a quite definite red colour. A 5 % solution of glucose gives none.

II. *Schiff's reaction.* Dixon and Harrison [1932] mention that glucosone gives this reaction. In the experiments to be described equal volumes of the solution under test and of the reagent were taken. The presence of 0·04 % of glucosone is easily detected. Glucose is entirely without action.

The presence of compounds other than glucosone might be indicated by either of these tests, but there would seem to be no other substance resulting from the oxidation of glucose which gives all three. The occurrence of positive reactions in all three cases affords, therefore, strong presumptive evidence of its presence and its formation has been assumed when this has occurred, though it is realised that the finding needs final confirmation by quantitative means.

Before applying the tests it was essential to remove all protein from the solution. This was accomplished by dialysing through cellophane. It was necessary to concentrate the dialysate before testing. Dixon and Harrison [1932] show that solutions of glucosone may be concentrated *in vacuo* in acid solution without appreciable loss. This has been confirmed.

A preliminary experiment carried out with mixed extracts of wet bottom diatoms and dried styles indicated that glucosone was produced. To 10 cc. of an extract of the diatoms made as described in a previous paragraph 0·25 g. of finely ground dried style and 0·5 g. glucose were added, the liquid was saturated with toluene and the mixture shaken at intervals until the style material was dissolved. It was then put into a wide-bottomed flask, in order to expose as large a surface to the air as possible and allowed to stand at air temperature for 48 hours. At the end of this time it was transferred to a small cellophane bag and dialysed against about 50 cc. of distilled water saturated with toluene. The water was changed at 12-hour intervals until, on testing with Fehling's solution, only a slight reducing action was obtained. The united dialysates were concentrated to 10 cc. *in vacuo* at 50–60°, after addition of 0·5 cc. of glacial acetic acid.

The three above-mentioned tests applied to 2 cc. samples of this concentrate gave in each case a positive reaction for glucosone. This has been repeated several times using extracts of both bottom and plankton diatoms, and similar results have been obtained using solutions of undried styles extracted from freshly-dug clams with care to retain the anterior ends carrying the diatoms, but, in the latter case, the glucosone reactions were never so strong as in those from solutions of the same concentration of dried style in diatom extract.

In order to determine to what extent the production of glucosone was to be attributed to the activity of each component of the mixtures, series of experiments were carried out in which each constituent was omitted in turn. The concentrations of the extracts and of glucose were the same as those in the experiments described above. The results obtained are summarised in Table I.

It is evident that the oxidation of glucose to glucosone by the combined extracts of style and diatoms depends upon the presence of the latter. The extract of diatoms alone can bring it about to only a small extent. The reaction proceeds in this respect in a manner precisely analogous to the oxidation of

Table I.

Mixture	*m*-Nitrophenylhydrazine	Fructose test	Schiff test
Style, bottom diatoms and glucose	Precipitate in 15 mins.	Strong colour	Coloration in 2 mins.
Style, plankton diatoms and glucose	Precipitate in 30 mins.	Strong colour	Coloration in 5 mins.
Style and bottom diatoms	Trace of precipitate in 30 mins.	Very slight reaction	Faint coloration in 6 mins.
Bottom diatoms and glucose	Slight precipitate in 30 mins.	Distinct coloration	Faint colour in 2 mins.
Plankton diatoms and glucose	Slight precipitate in 30 mins.	Fair coloration	Faint colour in 5 mins.
Style and glucose	No reaction	Trace of colour	No reaction

guaiacum or pyrogallol. In the absence of added glucose the glucosone reactions are very slight. Doubtless the trace which is found may be attributed to the glucose produced by the hydrolysis of carbohydrate in the diatom extract.

Presence of a dehydrogenase.

The nature of the oxidation of glucose brought about by the style with its added diatoms, consisting as it does of the removal of hydrogen from the glucose molecule, suggested that a dehydrogenase might be concerned.

An experiment was carried out with a mixture of extracts of bottom diatoms and dried style, made as described above, and arranged to detect the presence of such an enzyme. The liquid was centrifuged as soon as solution of the style material was complete, 5 % of glucose and 1 cc. of a 1/10,000 solution of methylene blue were added, and, after removal of a small sample for colour comparison, the mixture was immediately put into a Van Slyke [1917] CO_2 apparatus and pumped free from dissolved air. After standing *in vacuo* in this apparatus for 24 hours some decoloration of the methylene blue was evident by comparison with the sample removed before exhaustion, but it was difficult to estimate its degree because the blue was obscured by the yellow-green of the diatom pigment. After standing another 24 hours, during which the green colour of the solution became appreciably yellower, it was removed from the apparatus and heated to 98° for 2 minutes to inactivate the enzymes and so avoid risk of oxidation proceeding under the action of the oxidase during dialysis. After cooling the liquid was dialysed and the dialysate concentrated and tested as previously described. *m*-Nitrophenylhydrazine gave a definitely positive reaction for glucosone in 15 minutes and its presence was confirmed by the Schiff test.

Although it was not probable that the glucosone formed in this experiment was due to oxidation under the influence of the oxidase of glucose derived from the diatoms during the dissolution of the style material, since, as has been shown above, only traces are produced in this way, it seemed advisable to eliminate the possibility. The experiment was therefore repeated using plankton diatoms, which give a lightly coloured extract, in which decoloration of methylene blue can be easily followed, and an extract of dry style in water. The glucose was dissolved in the style extract and the diatom extract mixed with it immediately before addition of the methylene blue and exhaustion of air. After standing 6 hours *in vacuo* considerable decoloration had taken place; after 24 hours it was almost complete. 0·5 cc. more methylene blue solution was then added and, after re-exhausting, the solution was allowed to stand a further 24 hours during which further decoloration occurred. Strong reactions for glucosone were given by the concentrated dialysate obtained from this experiment.

Finally a similar experiment was carried out with a solution of undried styles, with their normal complement of attached diatoms, in water (about 3 g. styles to 15 cc. water). Only slight decoloration of the methylene blue took place, and all the glucosone tests were weak though definitely positive.

It was evident therefore that the combined extracts of style and diatoms contained a substance, or substances, which could reduce methylene blue and lead to the production of glucosone from glucose *in vacuo*. It remained to be determined whether these two reactions were connected and how far each constituent of the mixture was essential to them. To this end a series of experiments was carried out in which each constituent of the complete mixture was eliminated in turn. The experiments were maintained *in vacuo* for periods of 48 hours. Both bottom and plankton diatoms were used, but, since the results confirmed one another almost completely, they are not reported separately (Table II).

Table II.

Mixture	Decoloration of methylene blue	*m*-Nitrophenyl-hydrazine	Fructose test	Schiff test
Style, diatoms, glucose and methylene blue	Almost complete	Precipitate in 15 mins.	Strong colour	Colour in 6 mins.
Diatoms, glucose and methylene blue	Slight	Trace of precipitate in 30 mins.	Slight colour	Slight colour in 15 mins.
Style, glucose and methylene blue	Slight	Slight turbidity	Slight colour	No reaction
Style, diatoms and methylene blue	None	—	—	—
Glucose and methylene blue	Very slight	No reaction	Very slight colour	No reaction
Style, diatoms and glucose	—	Slight turbidity	Trace of colour	Very slight colour in 15 mins.

It is apparent from these results that the decoloration of methylene blue under the combined action of style and diatoms depends upon the presence of glucose; without it no decoloration occurs. Also that in the absence of methylene blue practically no formation of glucosone occurs, so that methylene blue is evidently essential as hydrogen acceptor for the dehydrogenation of glucose to proceed. It seems reasonable therefore to look upon the two reactions as connected by a dehydrogenase system transferring hydrogen from glucose to methylene blue. The full activity of this dehydrogenase system depends upon the presence of a diatom constituent. The decoloration of methylene blue and the glucosone reactions are all more pronounced in the case of the mixed extracts than in that of either alone. This diatom constituent, unlike that which functions as the autoxidisable component of the oxidase system, becomes inactive in dried diatoms which have been kept for some weeks. An extract of such dried diatoms in which dried styles and glucose had been dissolved did not decolorise methylene blue appreciably faster or more completely than a solution of the same concentration of styles and glucose in water, and the mixture led to only very slight glucosone reactions. Mann [1932] has shown that a hydrogen activator plays a part in the oxidation of glucose by the glucose dehydrogenase isolated by Harrison [1931] from liver. It is probable that such an activator is present in diatoms, but the evidence available does not exclude the possibility that the dehydrogenase is contained in the diatoms and an activator in the style. This point has yet to be investigated.

Discussion.

The observations that glucose is oxidised to glucosone by the activity of the crystalline style in contact with air under the influence of an oxidase system and also out of contact with air under that of a dehydrogenase system seem to demand a common explanation. The most probable one appears to be that the production of glucosone in solutions in contact with air is due to the activity of the dehydrogenase system and that the oxidase system serves merely as hydrogen acceptor to promote this activity, but proof that this is the case awaits the separation of the various substances concerned. That an oxidase system and a dehydrogenase system can thus interact has been shown by Harrison [1931] who was able to promote the oxidation of glucose in contact with air under the influence of his glucose dehydrogenase by means of an oxidase isolated from heart muscle. In this case cytochrome was necessary to serve as carrier between the two activating systems and, if the analogy be complete, some substance performing this function is to be looked for in the crystalline style or its accompanying diatoms.

It is clear that Harrison's glucose dehydrogenase differs essentially from the dehydrogenase of the crystalline style inasmuch as the one leads to the production of gluconic acid, the other to that of glucosone.

The only other enzymes bringing about the oxidation of glucose which have been described appear to be that of Müller [1929] isolated from *Aspergillus niger* and that which occurs in certain bacteria. The former behaves as an oxidase and produces gluconic acid. The latter is inactivated by toluene [Quastel and Wooldridge, 1927]. Both, therefore, differ from the dehydrogenase of the crystalline style.

This appears to be the first time that the production of glucosone as the result of animal, or partly animal, physiological activity has been described; it is indeed the only record of its occurrence as a biological product except that of Walker [1932] who detected it in a glucose medium in which a mould of the *flavus-oryzae* group of *Aspergilli* had been grown.

If the formation of glucosone from glucose is the only reaction brought about by the oxidising activity of the crystalline style, as seems to be the case, the suggestion that the CO_2 produced by clams during anaerobiosis might be attributed to that activity must be abandoned, since CO_2 is not a product of the reaction.

It is realised that the results which have been recorded in this paper are of a preliminary nature. Future work will be directed toward the separation of the several substances concerned in the reactions which have been described, the determination of the conditions governing their interactions, the quantitative identification of the product or products, and the effect of substituting other substrates for glucose.

Summary.

1. The oxidase system of the crystalline style of *Saxidomus giganteus* depends for its activity on at least two components, a peroxidase contained in the style itself and an autoxidisable substance contained in the diatoms constituting the food of the animals.

2. No acid is formed by the action of the oxidase system on glucose.

3. Glucosone appears to be formed by the action, but this needs quantitative confirmation.

4. A dehydrogenase system also occurs whose activity also depends on the joint action of a substance contained in the style and another contained in the diatom food material.

5. Glucosone also appears to be formed by the action of the dehydrogenase system on glucose in the presence of methylene blue *in vacuo*.

6. It is suggested that the oxidase system serves as hydrogen acceptor to promote the action of the dehydrogenase system.

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CLXXXIV. THE KERNEL-FATS OF SOME MEMBERS OF THE PALMAE: *ACROCOMIA SCLEROCARPA* MART. (GRU-GRU PALM), *MANICARIA SACCIFERA* GAERTN., *ASTROCARYUM TUCUMA* MART., *MAXIMILIANA CARIBAEA* GRISEB., *ATTALEA EXCELSA* MART. (PALLIA PALM), AND *COCOS NUCIFERA* LINN. (COCONUT).

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Up to the present only five examples of the kernel-fats of this important family have been examined by the methods now available for the quantitative estimation of the component fatty acids [Armstrong *et al.*, 1925; Taylor and Clarke, 1927; Hilditch and Vidyarthi, 1928; Collin and Hilditch, 1928; Saraiva, 1929; Heiduschka and Agsten, 1930], and, of these, two only have been submitted to oxidation with potassium permanganate, whereby a general idea of the glyceride composition may be obtained. In the present paper the relevant data for six more seed-fats of this family are communicated. Of these, five are new, in the sense that they have not hitherto undergone detailed analysis, and one, a commercial specimen of coconut oil, is of interest as a useful addition to the example already reported upon in an earlier paper [Collin and Hilditch, 1928].

Of the five new fats, those from *Acrocomia sclerocarpa* Mart., *Manicaria saccifera* Gaertn., and *Astrocaryum Tucuma* Mart., were available in sufficient quantity for a fatty acid analysis of the normal standard of accuracy and for a determination of their content of fully-saturated glycerides. The amounts of fat available from the kernels of *Maximiliana caribaea* Griseb. and *Attalea excelsa* Mart. were so small that it was only possible to make a very rough estimate of their component fatty acids. In the case of *Maximiliana caribaea* the fatty acid mixture was subjected to a single fractionation, and its general composition was then deduced from the analytical constants of the primary fractions, in the absence of the systematic refractionation which is strictly necessary when dealing with the complex mixture of acids characteristic of the Palmae seed-fats. In the other case, the kernel-fat of *Attalea excelsa*, the primary fractionation was controlled so as to correspond as closely as possible, in size of fraction and range of boiling-point, with an average primary fractionation of a Palmae kernel-fat, and by comparison of the two sets of analytical data a general idea of the composition of its component acids was obtained.

To guard against any possibility of loss of low-boiling esters during the vacuum distillation, a single-surfaced water-condenser was inserted between the Willstätter bulb and the Perkin triangle during the primary fractionation

and the first two refractionations. Apart from this alteration, both the ester fractionations and the acetone-permanganate oxidations follow the procedures adopted in the examination of coconut and palm kernel-fats [Collin and Hilditch, 1928].

Composition of the fatty acid mixtures.

The tendency of the various species within the palm family to synthesise fats of similar composition is shown very strikingly by a comparison of the fatty acid mixtures of the five new fats examined in this paper, (i) with each other, and (ii) with the recent analyses already mentioned of the component acids from coconut, palm kernel, cohune nut, murumuru and *Attalea funifera* kernel-fats.

Table I.

	% acids.							
	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic
Acrocomia sclerocarpa	7·8	5·6	44·9	13·4	7·6	2·6	16·5	1·6
Manicaria saccifera	5·3	6·6	47·5	18·9	8·2	2·4	9·7	1·4
Astrocaryum Tucuma	1·3	4·4	48·9	21·6	6·4	1·7	13·2	2·5
Astrocaryum Murumuru [Saraiva, 1929]	1·1	1·6	42·5	36·9	4·6	2·1	10·8	0·4
Maximiliana caribaea	(?)	5·0	47·0	21·0	9·0	(?)	(18)	
Attalea excelsa	(Similar composition to that of *Acrocomia sclerocarpa*)							
Cohune (*Attalea cohune*) [Hilditch and Vidyarthi, 1928]	7·5	6·6	46·4	16·1	9·3	3·3	9·9	0·9
Attalea funifera [Heiduschka and Agsten, 1930]	6·5	2·7	45·9	19·9	6·9	—	18·1	—
Coconut I [Collin and Hilditch, 1928]	7·9	7·2	48·0	17·5	9·0	2·1	5·7	2·6
Coconut II	7·8	7·6	44·9	18·1	9·5	2·4	8·2	1·5
Palm kernel [Collin and Hilditch, 1928]	2·7	7·0	46·9	14·1	8·8	1·3	18·5	0·7

In each case the same range of fatty acids is present, but each genus, as it were, asserts its individuality by slight variations in particular components while conforming to the same general type. The comparative constancy of the major constituent of the mixtures, lauric acid, is particularly noticeable and, similarly, capric, myristic and palmitic acids all occur within fairly narrow ranges. The apparent absence of caprylic and stearic acids from the fat of *Maximiliana caribaea* may well be attributed to incomplete separation of the mixture due to lack of material.

Glyceride structure of the kernel-fats of *Acrocomia sclerocarpa* Mart., *Manicaria saccifera* Gaertn. and *Astrocaryum Tucuma* Mart.

The determination of the content of fully-saturated glycerides in these three fats and the consequent disclosure of the extent to which the unsaturated acids are distributed throughout the glyceride molecules has demonstrated that the usual tendency towards even distribution, characteristic of kernel-fats, holds throughout this group. The numerical expression of the extent of dispersion of the unsaturated acids, the "association ratio" [Collin and Hilditch, 1929], is in fair agreement in all three cases with the corresponding values for coconut and palm kernel-fats and indicates that all these fats possess similar glyceride structures. The case for the absence of triolein from coconut and palm kernel-fats has been put in a previous paper [Collin and Hilditch, 1928] and applies

with equal force to the present fats. Assuming triolein to be absent, the glyceride composition of the fats may be expressed in terms of saturated glycerides, mono-unsaturated disaturated glycerides, and di-unsaturated monosaturated glycerides (the figures refer to mols. per 100 mols. of fat) (Table II).

Table II.

	Fully-saturated glycerides	Mono-unsaturated disaturated	Di-unsaturated monosaturated
Acrocomia sclerocarpa	69	21	10
Manicaria saccifera	82	11	7
Astrocaryum Tucuma	73	18	9

Acrocomia sclerocarpa (*Gru-gru*) *kernel-fat.*

This tree, known locally as the gru-gru palm, is indigenous to Central America and Brazil, although the specimen from which this sample of nuts was taken grew in Trinidad. The nuts were kindly supplied by the Department of Agriculture, Port of Spain, Trinidad, to whom we are also indebted for the specimens of *Manicaria saccifera* and *Maximiliana caribaea* nuts.

The nuts contained 26 % of kernel, which, by extraction with low-boiling light petroleum, yielded 44·4 % of a white soft fat having saponification equivalent 222·3, acid value 0·6, iodine value 17·1, unsaponifiable matter 0·45 % and M.P. (open tube) 24°. The corresponding mixed fatty acids, containing the unsaponifiable matter, had saponification equivalent 210·4, iodine value 18·0, and setting-point 22°. A quantitative analysis of the component fatty acids has not been published but various workers have determined the constants of the fat, and obtained values for the saponification equivalent varying between 220–230, iodine value 16–21, M.P. 21–32·5° and Reichert-Meissl value 5·7–7·2.

The component fatty acids, determined by the ester fractionation process as described [Collin and Hilditch, 1928], are given in Table III.

Table III.

	% acids	
	Weight	Mols.
Caprylic	7·8	11·5
Capric	5·6	6·9
Lauric	44·9	47·6
Myristic	13·4	12·2
Palmitic	7·6	6·3
Stearic	2·6	1·8
Oleic	16·5	12·5
Linoleic	1·6	1·2

Fully-saturated glycerides. The fat (99·6 g.) was dissolved in acetone and treated with potassium permanganate in the usual manner. On working up the oxidation product there were obtained 69·4 g. crude fully-saturated glycerides (iodine value 0·3). After further purification with boiling aqueous potassium carbonate, the crude product was separated into (*a*) 42·9 g. of saturated glycerides (saponification equivalent 208·3, acid value 1·4), (*b*) 20·6 g. of saturated glycerides (acid value 2·2), and (*c*) 3·84 g. of acid product. This corresponds with a content of fully-saturated glyceride in the original fat of 64·5 % by weight or of 69·1 mols. per 100 mols. of fat. It also indicates an "association ratio" in the mixed saturated-unsaturated glycerides of 1·29 mols. of saturated acid with 1 mol. of unsaturated acid.

Manicaria saccifera *kernel-fat.*

As in the case of *Acrocomia sclerocarpa*, the palms from which the nuts were obtained, although indigenous to Central America and Brazil, were grown in Trinidad.

The kernels formed 15 % of the nut and gave 57·7 % of a soft white fat on extraction with low-boiling light petroleum. The fat possessed the following analytical characteristics: saponification equivalent 221·8, acid value 0·6, iodine value 10·7, unsaponifiable matter 0·05 %, and M.P. (open tube) 27·1°. The mixed fatty acids, containing the unsaponifiable matter, had saponification equivalent 208·0, iodine value 11·4 and setting-point 24·3°. Again there is no quantitative analysis of the fatty acids on record, but there is a reference in the literature [Olien en Vetten, 1919] to a specimen of this fat with a saponification equivalent of 232·2, iodine value 19 and M.P. 30°.

The results of the detailed analysis of the mixed fatty acids from the present specimen are given in Table IV.

Table IV.

	% acids	
	Weight	Mols.
Caproic	Trace	—
Caprylic	5·3	7·8
Capric	6·6	8·0
Lauric	47·5	49·9
Myristic	18·9	17·4
Palmitic	8·2	6·7
Stearic	2·4	1·8
Oleic	9·7	7·3
Linoleic	1·4	1·1

Fully-saturated glycerides. Permanganate-acetone oxidation of the fat (25·9 g.) yielded 20·6 g. of crude fully-saturated material; this was resolved, by further purification, into (*a*) 19·50 g. saturated glycerides (saponification equivalent 214·5, acid value 0·5), (*b*) 0·71 g. saturated glycerides (acid value 2·9) and (*c*) 0·39 g. acid product. The content of fully-saturated glyceride is therefore 79·5 % (weight) or 82·3 % (mols.), corresponding to an association ratio of 1·16 to 1.

Astrocaryum Tucuma *kernel-fat.*

The natural habitats of this palm are Brazil, Central America and the West Indies, but the palms from which the fat now examined was obtained were grown in Malaya. The nuts were collected through the kindness of Messrs E. Boustead and Co. (who also supplied the nuts of *Attalea excelsa*) from the Malakoff Rubber Estates.

The decorticated kernels yielded 39·8 % of a white fat, rather harder than in the previous instances, of saponification equivalent 230·3, acid value 1·8, iodine value 15·8, unsaponifiable matter 0·4 % and M.P. (open tube) 30·3°. The mixed fatty acids had saponification equivalent 218·9, iodine value 16·6 and setting-point 26·8°. The values recorded in the literature vary between the following limits: saponification equivalent 225–226, iodine value 9·4–11·6, M.P. 30·5–35·5°.

The results of the fractionation of the esters of the mixed fatty acids in the usual manner are given in Table V.

Fully-saturated glycerides. Oxidation of the fat (98·6 g.) with potassium permanganate in acetone yielded 72·1 g. of crude saturated glycerides which

Table V.

	% acids	
	Weight	Mols.
Caprylic	1·3	1·9
Capric	4·4	5·6
Lauric	48·9	53·1
Myristic	21·6	20·6
Palmitic	6·4	5·5
Stearic	1·7	1·3
Oleic	13·2	10·1
Linoleic	2·5	1·9

were further separated into (*a*) 61·7 g. saturated glycerides (saponification equivalent 220·5, acid value 0·6), (*b*) 4·40 g. saturated glycerides (acid value 2·6) and (*c*) 2·24 g. acid product. The original fat thus contained 69·7 % (weight) or 72·8 % (mols.) of fully-saturated glycerides and its "association ratio" was 1·25 : 1.

Maximiliana caribaea *kernel-fat.*

This species is also indigenous to Central America and Brazil, but the nuts studied were grown in Trinidad. The nuts, which were obviously immature, contained 59 % of kernel of which only 4·8 % was fat. The fat had the following characteristics: saponification equivalent 236·8, acid value 20·2, iodine value 22·7, unsaponifiable matter 0·23 %. There are no previous references to it in the literature. As already explained, the amount of fat at disposal only permitted a rudimentary fractionation of the esters of the mixed fatty acids to be undertaken. The results suggested the following approximate proportions of component fatty acids (Table VI).

Table VI.

	% acids	
	Weight	Mols.
Capric	5	6
Lauric	47	50
Myristic	21	21
Palmitic	9	8
Stearic	?	?
Oleic } Linoleic }	18	15

Attalea excelsa (*Pallia palm*) *kernel-fat.*

This batch of nuts came from palms grown in Malaya, but the tree is a native of Brazil. The kernel formed only 3 % of the nut and yielded 62·6 % of fat with saponification equivalent 231·7, iodine value 18·2, M.P. (open tube) 25·5°. The mixed acids had saponification equivalent 218·6, iodine value 19·3. Previous workers [Wittka, 1925; Allan and Moore, 1925] give the following values: saponification equivalent 218–241, iodine value 8–16, M.P. 18–19°.

The primary fractionation of the mixed esters may be compared with the primary fractionation of the mixed esters of *Acrocomia sclerocarpa* (Table VII).

The similarity in range of boiling-point, weights and analytical constants between the two sets of fractions is sufficient indication that the two mixtures are broadly the same in composition. The fat from *Attalea excelsa*, however, is not so rich in the group of acids below C_{12} as *Acrocomia sclerocarpa* fat, and this is balanced by its increased content of myristic, palmitic and stearic acids.

Table VII.

	Attalea excelsa Mixed esters				*Acrocomia sclerocarpa* Mixed esters			
	B.P.	Weight %	S.E.	I.V.	B.P.	Weight %	S.E.	I.V.
1	55–88°	24	196·6	0·5	50–95°	29	188·6	0·3
2	88–90	25	217·6	0·7	95–105	30	217·7	0·5
3	90–130	31	252·8	17·2	105–130	18	245·6	15·8
4	Residue	20	295·9	60·3	Residue	23	291·7	64·2

Coconut oil.

The coconut oil used was taken from a refined commercial specimen supplied by Messrs Lever Brothers, Ltd., and had saponification equivalent 217·2, iodine value 9·7 and acid value 0·3. The experimental determination of the fatty acid mixture and the fully-saturated glycerides was carried out by Dr R. Child; the final composition of the component acids is given in Table VIII.

Table VIII.

	% acids	
	Weight	Mols.
Caproic	Trace	—
Caprylic	7·8	11·2
Capric	7·6	9·2
Lauric	44·9	46·5
Myristic	18·1	16·5
Palmitic	9·5	7·7
Stearic	2·4	1·8
Oleic	8·2	6·0
Linoleic	1·5	1·1

Fully-saturated glycerides. Permanganate-acetone oxidation of the fat (400 g.), yielded (*a*) 244 g. saturated glycerides (saponification equivalent 211, acid value nil), (*b*) 82 g. saturated glycerides (acid value 6·6), and (*c*) 57·6 g. acid product. The original fat therefore contained 80·7 % (weight) or 84 % (mols.) of fully-saturated glycerides. The corresponding "association ratio" of saturated to unsaturated acids in the mixed non-fully-saturated glycerides is 1·4 : 1.

This result is of interest when compared with those for the only other coconut-fat glycerides previously examined [Collin and Hilditch, 1928]. The specimen of fat then studied contained slightly more combined oleic and linoleic acids than the present one and, correspondingly, slightly less fully-saturated glycerides. The ratio of saturated to unsaturated acids in the non-fully-saturated glycerides was, however, the same in both cases. In seed-fats wherein unsaturated acids do not form more than about 40 % of the total fatty acids, the amount of fully-saturated glycerides is determined by the excess of saturated acids over and above the quantity necessary to link with unsaturated acids in mixed glycerides with an "association ratio" of about 1·3 to 1·4 : 1.

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CLXXXV. FATTY ACIDS FROM THE LARVA-FAT OF THE BEETLE *PACHYMERUS DACTRIS* L.

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When the nuts of *Manicaria saccifera* Gaertn. [Collin, 1933] were being prepared for extraction, many of the kernels were found to contain living grubs, which were identified as the larvae of the beetle *Pachymerus dactris* Linn. This insect has also been found in the kernels of the cohune nut (*Attalea cohune*) and is a common pest of these species [Hilditch and Vidyarthi, 1928]. The larvae had eaten all the fat-bearing endosperm, and in some cases had undergone metamorphosis into the beetles, which were boring their way through the hard shell of the nut. The fat from the larvae was extracted and obtained in sufficient quantity for a rudimentary analysis by the ester-fractionation method.

8·5 g. of semi-solid fat were obtained from 53 larvae (18 g.) by soaking them in acetone, drying, extracting with low-boiling petroleum and combining the acetone and petroleum extracts. This had saponification equivalent 260·7, iodine value 37·4 and acid number 3·2, and was analysed in the usual way by distilling the methyl esters of the "liquid" and "solid" acids (obtained by lead salt separation) from a 50 cc. flask with column into small receivers carried on a rotating support inside a vacuum desiccator. The quantities dealt with were so small that the accuracy of the analysis is not of a very high order.

The acids were separated into "solid" acids (57·5 %, iodine value 3·0) and "liquid" acids (42·5 %, iodine value 70·9): the calculated approximate composition of the mixed esters is given below:

	Esters % by weight
Lauric	24
Myristic	21
Palmitic	8
Oleic	32
Linoleic	3
Stearic, oleic or linoleic (present in residual fractions)	12

Comparing these figures with those for the kernel fat of *Manicaria saccifera* [Collin, 1933], it appears that the acids of lower molecular weight (of which lauric acid is the chief) are present in the larva-fat in only about half the amount in which they occur in the kernel-fat, while oleic and linoleic acids probably form about 40 % of the mixed acids in the larva-fat, as compared with only 11 % in the kernel-fat. This rather suggests that the insect has derived its fat partly by direct assimilation of the preformed vegetable fat, and partly by synthesis from carbohydrate (or other non-fatty) components of the kernel. If this be the case, it would appear that the development of fat in insects may follow a course not very different from that which takes place in the larger land vertebrates, such as the pig.

Unfortunately there is little information in the literature on insect fats. The cocoon of the silkworm, *Bombyx mori* Linn., contains about 25 % of fat

consisting of mixed glycerides of palmitic, oleic, linoleic and linolenic acids, and the mixed fatty acids include about 25 % of saturated (palmitic and stearic) with about 22 % oleic, 38 % linoleic and 15 % linolenic acids [Tsujimoto, 1908; 1916; Suzuki and Yokoyama, 1928]. The only other instances of larva-fats are those from two insects of unknown origin [Desvergnes, 1920], the mixed acids of which had a mean molecular weight of 263–269 and contained unsaturated acids (50–60 %) with iodine values of 140 and 95. The general characteristics of a few fats of insects belonging to the Coleoptera, Diptera and Orthoptera have been reported, from which it appears that the major components are oleic and linoleic acids and that, although palmitic acid is probably also present, acids of lower molecular weight are absent. An exception to this statement is, however, found in the fat of *Pemphigus* species (Aphidae), the acids of which are reported by Schultz [1922] to have a mean molecular weight of 218 and to include butyric, caprylic and lauric as well as palmitic acids.

On the whole, it seems likely that insects, in the larval as well as mature state, lay down fats somewhat similar in type to those produced by mammals, and that, like the latter, they can assimilate fats present in their diet and also synthesise fat from other constituents of the food. More complete study of insect fats than has hitherto been made might well be of interest from a biochemical standpoint.

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CLXXXVI. THE COMPONENT FATTY ACIDS OF RAT BODY FATS.

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Detailed quantitative information upon the composition of the fatty acids combined in the depot fats of animals is abundant in the cases of the ox, sheep and pig, but is otherwise still scanty, except in a few isolated instances, such as the loin fat of the reindeer [Baughman *et al.*, 1929] and the body fat of the horse [Heiduschka and Steinruck, 1921; Grossfeld, 1931]. These two fats appear to fall into the same category as the tallows and lards, in that their component acids contain about 30 % (weight) of palmitic acid and substantial, but variable, proportions of stearic acid, the amount of the latter varying more or less inversely with that of the oleic acid present [*cf.* Banks and Hilditch, 1932]. Of bird depot fats, those of the goose [Bömer and Merten, 1922; Grossfeld, 1931] and the hen [Grossfeld, 1931] have been examined by resolution into "solid" and "liquid" acids by lead salt separation (Twitchell), or by oxidation (Bertram). The bird fats seem to belong to a different type from the animal fats mentioned, since the analyses quoted indicate the presence of only small amounts of stearic acid (usually not more than 5 %), and the proportion of palmitic acid is also lower (20–25 %). These differences, which are confirmed and extended, in the case of hen fats, by studies by the ester-fractionation method now proceeding in this laboratory, point to the desirability of collecting detailed quantitative data for the depot fats from a much wider variety of animals than that dealt with up to the present.

As a contribution to this end, we are able to communicate the results of analyses, by the ester-fractionation process, of four rat body fats and of the perinephric fat from wild rabbits (the latter analysis having been carried out by Mr J. R. Vickery in 1928, but not hitherto published). Burr and Burr [1930] pointed out that rats appear not to lay down linoleic acid in their reserve fats and suggested that they are unable to synthesise linoleic acid; according to these authors, the absence of fats containing linoleic acid from the diet causes the animals to develop the condition known as "scaly tail." Gregory and Drummond [1932] studied the depot and liver fats of rats fed on various diets by examining the analytical characteristics of the mixed "liquid" or mainly unsaturated acids obtained from the Twitchell lead salt separation. They confirmed Burr and Burr's observations that, with fat-free diets, the body fats were substantially devoid of linoleic acid, but found that this acid was present in the liver fats, from which it would follow that rats are able to synthesise this acid but do not deposit it in the reserve fat.

This peculiarity, the extensive use made of rats in nutritional experiments, and the desire already mentioned to extend the range of information on animal depot fat component acids, made us welcome an offer by Prof. Drummond to

place at our disposal, for study by the ester-fractionation method, specimens of depot fats collected from rats which had been fed (*a*) on mainly fat-free diets and (*b*) on diets supplemented by cod-liver oil. We are further indebted to Prof. Drummond for the information which follows regarding the origin of these four rat fats.

Each sample represented the whole of the adipose tissue fat dissected out from the subcutaneous, perinephric and mesenteric areas of a number of animals. Each rat yielded in all 5–6 g. of fat, and the total amount of available material in each specimen varied from 40 to 70 g. The latter quantity would have been sufficient for an accurate determination of a simple mixture of palmitic, stearic, oleic and linoleic acids; it will be seen, however, that the analyses were complicated by the apparent presence of small proportions of an unsaturated fatty acid of lower molecular weight than oleic acid (probably palmitoleic acid). Owing to this, it would be necessary to work with much larger quantities of material than were available in order to obtain data of the desired order of accuracy. The figures given for palmitic, stearic and linoleic acids, however, are probably very close to the truth, whilst that for oleic acid must be regarded as uncertain to within perhaps 3 or 4 units %. Fortunately, the general and characteristic features of the component acids are quite evident in spite of the minor uncertainties thus unavoidably present in some of the numerical data.

Three samples of rat fats were originally supplied.

Fat A was derived from rats fed on "fat-free" diets, but most of the animals had received daily doses of about 15 mg. of ethyl laurate as a carrier of vitamin A or carotene over periods varying from 3 to 7 weeks.

Fat B was from rats fed on different diets, none of which contained unsaturated fats. Most of these animals were on very low fat diets, but in some cases 10 % of the diet consisted of hydrogenated cottonseed oil; nearly all had supplements conveyed in ethyl laurate (as in A).

Fat C was from rats which had received diets containing from 2 to 15 % (in most cases 5 %) of cod-liver oil over periods of 10 weeks.

In view of the small amount of C available (*ca.* 40 g.), and of the interesting results disclosed, Prof. Drummond kindly undertook a special experiment in which 12 rats were fed for 12 weeks on a ration of "Glaxo casein" 20 %, rice starch 73 %, salt mixture 5 % and cod-liver oil 2 %. The resulting fat (D, 70 g.), however, did not reproduce the characteristics of fat C, possibly owing to the low proportion of cod-liver oil used.

Each fat was saponified, and the mixed fatty acids were divided into "solid" (mainly saturated) and "liquid" (mainly unsaturated) acids by the usual lead salt separation from alcohol (in presence of 0·5 % acetic acid). Each group of acids was then converted into methyl esters and fractionated under low pressure. The fractionation data are quoted in full (Table III) in order to make clear the basis of the deductions as to the composition of the mixed acids.

The general characteristics of the fats, all of which were almost entirely colourless and not completely solid at room temperature, may first be recorded (Table I).

Table I.

Fat	Diet	Saponification equivalent	Iodine value	Acid value	Setting-point of mixed fatty acids
A	"Fat-free"	281·0	60·5	34·8	37·2°
B	Mainly "fat-free"	283·3	57·3	17·0	38·2
C	2–15 % cod-liver oil	284·2	74·6	18·9	36·2
D	2 % cod-liver oil	280·3	57·4	20·0	39·6

The proportions of "solid" and "liquid" acids obtained from the mixed fatty acids of each fat in the preliminary lead-salt separation are given in Table II.

Table II.

Fat	"Solid" acids g.	"Solid" acids %	"Liquid" acids g.	"Liquid" acids %
A	14·3	30·7	32·3	69·3
B	18·9	33·9	36·8	66·1
C	9·7	28·4	24·5	71·6
D	21·2	35·3	38·8	64·7

The details of the ester-fractionations are given in Table III. The last fraction in each case represents the residue left in the distillation bulb. In the distillations of the "liquid" esters the residues, after saponification, are freed from unsaponifiable matter, and the equivalents and iodine values of the recovered acids (free from unsaponifiable) are re-determined in order to estimate

Table III. *Fractional distillation of methyl esters of the "solid" and "liquid" acids.*

No.	"Solids" g.	"Solids" Sap. Eq.	"Solids" I.V.	"Liquids" g.	"Liquids" Sap. Eq.	"Liquids" I.V.	
			From fat A ("fat-free" diets).				
1	3·04	264·4	3·2	5·67	268·9	75·9	
2	4·11	269·8	4·0	4·49	279·2	85·1	
3	3·54	273·0	5·9	4·90	285·7	86·8	
4	2·97	282·0	12·0	5·52	290·2	87·8	
5	—	—	—	4·77	294·0	88·5	
6	—	—	—	3·40	306·3	88·7	
	13·66	—	—	28·75	(296·6)	(88·6)	(Unsap.-free)
			From fat B (mainly "fat-free" diets).				
1	3·33	264·8	2·0	4·41	273·0	75·5	
2	3·15	268·5	2·5	4·55	282·4	82·1	
3	2·79	269·1	3·4	4·38	286·7	84·2	
4	3·86	271·8	4·2	5·39	292·3	84·3	
5	2·64	281·5	11·3	4·82	294·1	85·6	
6	—	—	—	5·02	296·3	85·8	
7	—	—	—	4·04	305·0	89·3	
	15·77	—	—	32·61	(296·7)	(88·0)	(Unsap.-free)
			From fat C (2–15 % cod-liver oil in diets).				
1	2·60	266·9	1·7	3·67	276·5	77·6	
2	1·84	269·1	3·4	3·80	286·3	88·4	
3	2·35	270·9	4·7	3·90	290·3	88·2	
4	2·74	281·0	11·3	3·84	296·0	95·7	
5	—	—	—	5·06	300·5	104·6	
6	—	—	—	3·95	320·4	155·8	
	9·53	—	—	24·22	(314·0)	(150·8)	(Unsap.-free)
			From fat D (2 % cod-liver oil in diets).				
1	2·94	266·2	0·9	12·12	277·4	80·8	
2	3·70	267·7	1·9	4·62	288·0	87·1	
3	3·60	269·6	2·1	4·52	289·6	87·4	
4	4·83	271·9	3·6	4·18	295·6	88·1	
5	4·31	280·3	9·9	4·66	294·7	88·8	
6	—	—	—	6·12	303·8	93·9	
	19·38	—	—	36·22	(295·5)	(91·4)	(Unsap.-free)

the amount of unsaponifiable material present. The values so obtained (stated in terms of the corresponding methyl esters) are added in brackets in Table III. (All the unsaponifiable matter passed, in solution in alcohol, into the "liquid" acids.)

When the composition of the lower-boiling fractions of the "liquid" methyl esters from fats A, B and C was estimated on the assumption that no unsaturated esters were present of lower molecular weight than the C_{18} ester-mixtures (of respective iodine values 88·5, 85·8 and 95·7), it was found that the calculated mean equivalents of the saturated material in these fractions were much lower than that of methyl myristate. The lowest-boiling fraction of "liquid" esters from fats which have undergone oxidative rancidity often exhibits this feature, although not to the same extent as in the present instances; moreover, the rat fats now described had not become oxidised appreciably. An alternative explanation lay in the presence of an unsaturated acid of lower molecular weight than oleic acid and, although the amount of material at our disposal was insufficient to permit of any positive identification of such acid, some indirect evidence was obtained by modifying the fractional distillation of the "liquid" esters from fat D.

In this instance, a considerably larger first (lowest-boiling) fraction than usual was collected (12·12 g., or about one-third of the whole, and containing nearly all the esters of lower molecular weight than methyl oleate-linoleate). The amount (8·7 g.) not required for direct analysis was dissolved in acetone and oxidised with potassium permanganate in order to convert all unsaturated esters into acidic products; the residual neutral product was then re-submitted to the same oxidation process. After the double oxidation, the remaining neutral ester (0·97 g.) still possessed a small iodine value (5·1) and the fatty acids combined therein (after boiling with water to remove traces of nonoic, azelaic, *etc.*, acids) were solid and had a mean molecular weight somewhat higher than that of myristic acid. After correcting for the small residual iodine value of the product obtained from the oxidations, the corresponding weight of saturated ester present in the original fraction (12·12 g.) was 1·3 g. Assuming this to be methyl myristate (although actually small proportions of methyl palmitate were probably also present), the mean molecular weight of the unsaturated esters also present was about 282; calculated in terms of a mixture of methyl palmitoleate and oleate-linoleate (of mean iodine value 88·8), this corresponds with the possible presence of 4·8 g. methyl palmitoleate and 6·0 g. methyl oleate-linoleate in this fraction.

On this basis, the component fatty acids of fat D were made up approximately of myristic (4 %), palmitic (30 %), stearic (2·5 %), palmitoleic (?) (8·5 %), oleic (53 %) and linoleic (2 %) acids.

Since, therefore, the presence of small amounts of an unsaturated acid of lower molecular weight than oleic acid was definitely indicated, we have calculated the component fatty acids of all four fats on the basis that the "liquid" ester fractions are made up of methyl myristate, palmitoleate and oleate (or oleate-linoleate). The results are given in Table IV.

We are aware that the impossibility of definitely identifying an unsaturated acid of lower molecular weight than oleic acid in the present series renders the foregoing calculation somewhat unsatisfactory, and we therefore add, for fats A, B and C (Table V), the results of the alternative calculation, in which the absence of unsaturated acids with less than 18 carbon atoms in the molecule is assumed. This, however, involves the presence of small amounts of saturated acids (lauric and even capric) of lower molecular weight than myristic acid;

and we have not been able to detect the presence of any acids of lower molecular weight than myristic acid.

Table IV. *Component fatty acids of rat body fats A, B, C, D.* (*Assuming the presence of palmitoleic acid.*)

Diet ...	A "Fat-free" %	B Mainly "fat-free" %	C 2–15 % cod-liver oil %	D 2 % cod-liver oil %
Myristic acid	5	4·5	5	4
Palmitic acid	24	28	23	30
Stearic acid	3	2	2·5	2·5
Palmitoleic (?) acid	8	7	5·5	8·5
Oleic acid	58	58·5	51·5	53
Linoleic acid	2	—	4	2
C_{20-22} unsaturated acids*	—	—	8·5	—

* In fat C the fractionation data clearly indicated the presence of highly-unsaturated acids of the C_{20} and C_{22} series in the highest-boiling and residual fractions of the "liquid" esters; their amount was approximately calculated by the same means as that employed in similar cases which have been encountered in pig body fat analyses [Banks and Hilditch, 1932].

Table V. *Component fatty acids of rat body fats A, B, C.* (*Assuming absence of any unsaturated acids of lower carbon content than* C_{18}.)

Diet ...	A "Fat-free" %	B Mainly "fat-free" %	C 2–15 % cod-liver oil %
Myristic and lower saturated acids	4·5	4	5
Palmitic acid	24	28	23
Stearic acid	3	2	2·5
Oleic acid	66·5	66	54·5
Linoleic acid	2	—	6·5
C_{20-22} unsaturated acids	—	—	8·5

Discussion.

It will be understood, from what has already been said, that the values given for the amounts of oleic acid present depend upon whether a palmitoleic acid, or even some unsaturated acid of still lower molecular weight, is also present; the figures for oleic acid in Tables IV and V together represent, however, the extreme possible limits. Apart from this uncertainty, a number of points of interest emerge from the analyses.

Body fats (A and B) of rats fed on mainly fat-free diets. The results amply confirm the observations of the previous investigators, namely, that rats receiving no linoleic acid in their fatty diet deposit reserve glycerides from which that acid is almost or completely absent.

A feature which interests us especially, however, is the relative amounts of palmitic acid and of stearic acid present in these fats as compared with the corresponding proportions in the body fats of pigs, oxen and sheep. The proportion of palmitic acid is, indeed, of much the same order in all cases: expressed in molar percentages, the united amounts of palmitic and myristic acids (the latter being 4–5 % in each case) in the four rat fats lie between 30 and 35 %, a figure which is almost identical with that observed throughout a large series of lards and tallows [*cf.* Banks and Hilditch, 1931; 1932]. The proportions of stearic acid are, on the contrary, strikingly different in the body fats of the rat and of pigs, sheep and oxen. In the latter group stearic is definitely a major

component acid, occurring in widely varying amounts. In the rat body fats it is equally clearly a minor component and occurs in proportions relatively small compared with those of palmitic or oleic acid.

In other words, the component acids of the rat body fats consist essentially of 25–30 % of palmitic acid, the remainder (apart from small proportions (4–5 %) of myristic and 2–3 % of stearic acid) being made up entirely of unsaturated acids, in which oleic acid predominates, and from which linoleic acid is practically absent. In consequence, about 65–70 % of the rat fat component acids belong to the unsaturated (oleic) series, whereas in lards and tallows the unsaturated acids vary from less than 40 %, and rarely exceed 55 %, of the mixed fatty acids.

Fully-saturated glycerides were only present in very small proportions in either fat, specimen A containing about 2·5 % and specimen B about 3·5 %. The fatty acids present in the fully-saturated components had mean molecular weights of 260 (A) and 258 (B) (palmitic acid, mol. wt., 256), so that these consisted almost entirely of tripalmitin. This is, of course, another difference from the depot fats of pigs, sheep and oxen, since the latter, in addition to containing greater amounts of stearic acid, also contain fully-saturated components in proportions increasing with those of the stearic acid present, and consisting for the most part of mixed palmitostearins [Banks and Hilditch, 1931; 1932].

Body fats (C and D) of rats fed on diets which included cod-liver oil. The specimen C, which was derived from rats whose diet had contained from 2 to 15 % of cod-liver oil, was differentiated from the rest by the presence of a certain amount (about 8 %) of the highly-unsaturated acids of the C_{20} and C_{22} series which are present in cod-liver oil. Apart from this, the remaining acids appeared to be present in much the same relative proportions as when the diet of the animals was mainly fat-free, but the presence of linoleic acid had become definitely noticeable (4 %). Nevertheless, the linoleic acid content is surprisingly small when compared with that of the deposited unsaturated C_{20-22} acids, since each type of acid is present in much the same proportions in cod-liver oil [Guha *et al.*, 1930].

This feature is also peculiar in view of the observation of Gregory and Drummond [1932] that the body fat of rats fed on diets including olive oil contained unsaturated acids of which linoleic acid formed from 7 to 10 % (*i.e.* practically the same proportion as in olive oil itself). With the olive oil diets used by these workers, there was, moreover, a considerable decline in the saturated acid content of the body fats, namely, from 32 % (fat-free diet) to 22 % (12 % olive oil in diet) and 11 % (50 % olive oil in diet). The fact that, in fat C, the total content of saturated acids (30·5 %) was only slightly less than that in the fats from animals on mainly "fat-free" diets (32–35 %), suggests either that the added cod-liver oil was not freely utilised, or, more probably, that the average amount of cod-liver oil in the diets (about 5 %, *cf.* p. 1376) was insufficient to produce any marked alteration in the body fat. In the case of the pig, for example, addition of cottonseed oil to the diet does not cause definite modification of the body fat until the oil forms more than 4 % of the diet [Ellis *et al.*, 1931].

The body fat D (from rats which had received a diet including only 2 % of cod-liver oil) was of practically the same composition as fats A and B, and showed no increase in linoleic acid and no perceptible amount of the higher unsaturated acids. The only indication of the presence of the latter was a slightly increased iodine value (93·9) in the residual fraction of "liquid" esters

(Table III), but this was not accompanied by any perceptible increase in the equivalent (295·5) of these esters (after correcting for unsaponifiable matter). In this experiment, therefore, the characteristic cod-liver oil acids did not appear in the depot fat.

The perinephric fat of the (wild) rabbit. We are able to add some details as to the adipose tissue fat of another rodent, from an ester-fractionation analysis (made in this laboratory some years ago by Mr J. R. Vickery) of the component fatty acids of the perinephric fat from wild rabbits. This was a yellow-coloured, very unsaturated fat, almost liquid at the ordinary temperature, with a saponification equivalent of 285·2 and an iodine value of 124·0. The component acids were made up of myristic (4·5 %), palmitic (23 %) and stearic (4 %) acids, the remainder (68·5 %) consisting of unsaturated acids (apparently almost wholly of the C_{18} series) with a mean iodine value of 189·3. The latter acids yielded solid hexabromo-addition products equivalent to a content of 9 % of linolenic acid in the total fatty acids; it is therefore quite possible that the total amount of linolenic acid in the mixed acids approached 15–20 %, since the yield of "insoluble hexabromides" usually corresponds with not more than about 50 % of the linolenic acid present.

This rabbit perinephric fat closely resembles the rat fats in its proportion of palmitic, stearic and myristic acids and in its general content of about 30 % saturated and 70 % unsaturated acids. It is also similar in its fully-saturated glyceride content, namely, about 6–7 %, almost wholly tripalmitin (the latter triglyceride was also obtained from rabbit fat by fractional crystallisation by Klimont [1912]). The nature of the unsaturated acids in rat fats and in rabbit fats is, however, wholly dissimilar: whereas, in that of the rat, linoleic acid is practically absent, the rabbit fat described contained large amounts of this component (35–50 % of the mixed acids) and also appreciable quantities of linolenic acid.

Summary.

The body fat of the rat belongs to a group in which the component acids are made up of palmitic (25–30 %), myristic (4–5 %) and small amounts of stearic (2–3 %), the remainder (about 65–70 %) consisting chiefly of oleic acid, apparently with small quantities of a palmitoleic acid; linoleic acid is, however, almost entirely absent.

When the diet of the animals includes cod-liver oil, some of the highly-unsaturated acids of the C_{20} and C_{22} series may occur in the depot fats, but otherwise little modification occurs, and the proportion of linoleic acid is only slightly increased.

The perinephric fat of a wild rabbit contained similar amounts of myristic (4·5 %), palmitic (23 %) and stearic (4 %) acids, but the unsaturated acids (68·5 %) differed widely from those of the rat fats, linoleic acid being the main component, while considerable amounts of linolenic acid were also present.

Fully-saturated components (almost wholly tripalmitin) were present only in small proportions in the body fats of either the rat (2–3 %) or the rabbit (6–7 %).

There seem to be at least two well-defined groups of depot fats characteristic of land (*i.e.* non-aquatic) animals.

(*a*) Fats with component fatty acids containing about 30–35 % saturated (usually 25–30 % palmitic) acids and for the rest unsaturated acids of varying type (oleic, linoleic, *etc.*). In these stearic acid is a minor component (not exceeding about 5 %), and fully-saturated components are only present in small

proportions and consist mainly of tripalmitin. The rodent body fats discussed in this paper, and probably also bird depot fats, fall in this category.

(*b*) Fats with component fatty acids made up of about 25–30 % palmitic and small amounts of myristic acids, the remaining 65–70 % consisting of stearic, oleic and linoleic acids. In this group (to which belong the depot fats of the pig, ox, sheep, horse, reindeer) the proportion of stearic acid is variable but usually important. The greater the amount of stearic acid (with correspondingly less oleic acid), the greater is the proportion of fully-saturated glycerides in the fat; in this group, these consist for the most part of mixed palmitostearins.

The persistence with which the palmitic acid content remains in the neighbourhood of 25–30 %, and that of myristic acid at about 4–5 %, throughout both groups is noteworthy.

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CLXXXVII. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

IV. THE ACTION OF PREPARATIONS OF ANIMAL PROTEOLYTIC ENZYMES, AND SOME OBSERVATIONS ON THE NATURE OF THE HORMONE.

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In Part III of this series Gulland and Macrae [1933] described the action of preparations of some plant proteolytic enzymes on the oxytocic hormone. They showed that preparations of yeast dipeptidase, aminopolypeptidase and proteinase, and of papain, contained an inactivating enzyme which had its p_H optimum at 7·4. This enzyme, which was not identified, reduced the oxytocic activity to 0·7 % of its initial value.

In continuing the investigation of the action of proteolytic enzymes, attention was then turned to those of animal origin. It was stated by Dudley [1919] and by Thorpe [1926] that pancreatic trypsin destroys the hormone. Dale and Dudley [1921] also recorded a destruction by intestinal erepsin. Freudenberg *et al.* [1932] found that pepsin and erepsin did not attack the hormone, but that it was destroyed by trypsin[2] and also by papain. No details of the nature of the erepsin used were given by these latter authors, but in view of the date of the investigation it may be presumed to have been more nearly enzymically homogeneous than that of Dale and Dudley.

Apparently on the basis of these results, Freudenberg and his collaborators concluded that the hormone has either a high molecular weight or is bound to a high-molecular carrier of the protein group. They also stated that the hormone is evidently of the same type as insulin. These views are in opposition to the conclusions of Smith and McClosky [1924] and of Kamm [1928] which were based on comparisons of rates of dialysis and on the relative permeability of the membranes used to the hormone, adrenaline and dyestuffs. Smith and McClosky observed that the diffusibility of the hormone was about the same as that of methylene blue, and Kamm suggested 600 as the molecular weight. He also concluded that the oxytocic hormone is more complicated than adrenaline, but simpler than insulin or the parathyroid hormone.

Unpublished experiments of our own on the pressure dialysis through pyroxylin thimbles of solutions of the hormone which had undergone a purification by charcoal adsorption [Gulland and Newton, 1932] led to the view that the hormone is probably not proteinoid in character. Consequently the destruction of the hormone by trypsin preparations was probably not due to trypsin

[1] Lister Institute Research Student in Biochemistry.

[2] It is assumed that activated trypsin was intended, since Waldschmidt-Leitz and Purr [1929] have shown that trypsinogen (unactivated trypsin) has no proteolytic activity.

itself, as believed by the investigators mentioned above, but was the result of one or more accompanying enzymes. In arriving at this conclusion we were also influenced by the observation in Part III that the inactivation by papain preparations, which was quoted by Freudenberg *et al.* [1932] in support of the protein nature of the hormone, is in fact due to another enzyme, which is not a papainase and has no action on gelatin.

When this conclusion was investigated experimentally, it was found that the hormone was extremely rapidly inactivated by a trypsin preparation supplied by British Drug Houses, Ltd. The shape of the p_H-activity curve and the position of the p_H optimum (Fig. 1) resembled in general those which characterise the action of activated trypsin on proteins. Markedly different results,

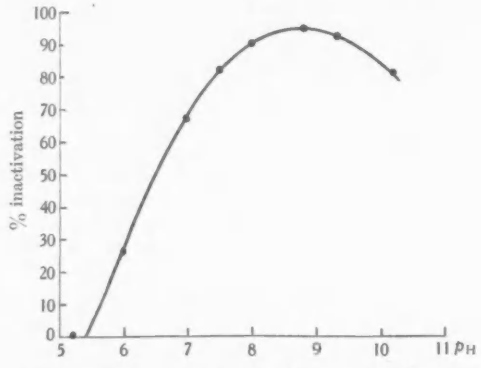

Fig. 1. p_H-activity curve of inactivation by trypsin preparation.

however, were obtained with the same weight of an 11-year old Kahlbaum preparation of trypsin under the same conditions; the hormone was inactivated, but only very slowly. The possession of these widely different trypsin preparations offered the opportunity of comparing directly their tryptic activity with their power of inactivating the hormone, and thus of determining whether activated trypsin itself has any action on the hormone.

By following with the titration method the early stages of the hydrolysis of gelatin by these enzyme preparations, it was shown (Table III) that the tryptic activity of the B.D.H. preparation was about 50 times stronger than that of the Kahlbaum preparation; by considering only the early stages of the hydrolysis it was permissible to discount for practical purposes the effects due to ereptic activity, which also was considerably higher in the B.D.H. preparation.

Two parallel experiments under identical conditions were then carried out to compare the powers of inactivating the hormone of the B.D.H. trypsin preparation on the one hand and of 50 times its weight of the Kahlbaum preparation on the other. If activated trypsin is responsible for inactivating the hormone, the inactivation in these two experiments should have been about the same. In actual fact, however, the inactivation by the B.D.H. preparation was many times more rapid than by the Kahlbaum preparation (Fig. 2). Thus activated trypsin itself is not responsible for the inactivation of the hormone by trypsin preparations, but this is caused by an enzyme with a p_H optimum at 8·8 (Fig. 1). This enzyme is not identical with the inactivating enzyme of yeast

and papain (Part III), and it is improbable, though not quite excluded, that they are the plant and animal variants of the same type of enzyme (identical substrates), because the enzyme of the trypsin preparation reduced the oxytocic value to 0·3 % (Table I) as compared with the value of 0·7 % in the experiments with the yeast-papain enzyme. As in the case of the activity remaining after the action of the latter enzyme, our present opinion is that the residual activity of 0·3 % is due to a derivative of the hormone produced by enzymic action.

It has thus been shown in this publication and in Part III that the apparent inactivation of the hormone by enzymes of which the characteristic substrates are proteins is not brought about by those enzymes themselves but by unidentified enzymes which accompany them. These facts seem to dispose of the contention that the hormone is proteinoid in character.

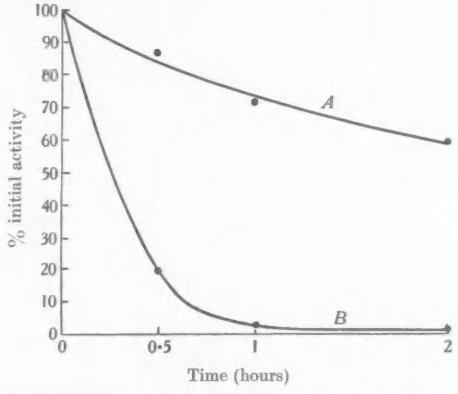

Fig. 2. Comparison of inactivation by weights of Kahlbaum trypsin (*A*) and B.D.H. trypsin (*B*) preparations having the same tryptic activity.

From the same experiments it is apparent that the sole support for the view advanced by others that the hormone is peptide in character lies in the fact that the enzymes which inactivate it accompany proteolytic enzymes. We are not opposed to the conception that the hormone molecule contains peptide linkages, but at present there is no evidence on this point.

Freudenberg *et al.* [1932] observed that pepsin did not attack the hormone. Presumably, although this is not stated, their experiments were made at or about the strongly acid p_H optimum of pepsin. As the beginning of a general study of the effect on the hormone of the enzymes of animal tissues, a proteolytically potent specimen of pepsin was examined at p_H 4·4 and p_H 7·2 for the presence of other enzymes which might attack the hormone. In neither case was any diminution in oxytocic strength observed. A bone phosphatase preparation also was tested at p_H 7·8 and found to be quite inactive towards the hormone.

From what has already been stated and from a general consideration of the problem, it is evident that a series of favourable results in a definite and somewhat prolonged sequence of investigations is required for the complete experimental demonstration that any given enzyme does inactivate the hormone. In the proof that an enzyme does not inactivate the hormone, a part or even most

of this sequence may be omitted. Apart from haphazard guesswork, the suggestion that a particular well-defined enzyme inactivates the hormone must be formulated as a result of facts observed in one or more of the following modes of attack: from a systematic examination of the effects of enzymes in general; from predictions of the presence of chemical groups as a result of the study of the action of chemical reagents; from the similarity of the p_H-activity curves of the inactivating enzyme acting on the hormone and of the suspected enzyme[1] acting on its known substrate. The last-mentioned source suffers from the disadvantage that the shape and optimum of a p_H-activity curve may vary with change of substrate. The stages of the demonstration are tabulated below.

(1) The discovery of the rate at which the hormone is inactivated by the preparation of the inactivating enzyme, and the determination of the p_H-activity curve.

(2) The examination of the action of the inactivating enzyme on the substrate of the suspected enzyme. If the degree of activity permits, the determination of the p_H-activity curve.

(3) The examination of the action of an authentic preparation of the suspected enzyme on the hormone, including the determination of the rate of action and of the p_H-activity curve for comparison with those in (1).

(4) Careful estimations of the activities of (*a*) an authentic preparation of the suspected enzyme, and (*b*) of the inactivating enzyme preparation towards the substrate of the suspected enzyme. This will permit a numerical comparison of the amounts of the suspected enzyme present in its authentic preparation and in the preparation of the inactivating enzyme.

(5) The demonstration of equal inactivation of the hormone under the same conditions by weights of preparations of the suspected enzyme and inactivating enzyme, which it is estimated would cause the same degree of inactivation if the suspected enzyme and inactivating enzyme were identical.

It was shown in Part II [Gulland, 1933] that two out of the three well-defined inactivations of the hormone which occur in presence of nitrous acid are probably due to nitrous acid itself. The rates of these inactivations and the conditions under which they occurred were not inconsistent with the view that the slower, which required a large excess of the reagent, was due to the interaction of nitrous acid with a guanidine group, and that the more rapid was the result of its interaction with an amino- or basic imino-group. A preliminary consideration from the enzymic aspect was not opposed to this conception, which was therefore examined more fully.

The position at a relatively alkaline reaction of the p_H optimum of the inactivation of the hormone by the B.D.H. trypsin preparation was not far removed from that of arginase. Moreover, this preparation contained a small, but definite, arginase activity, which was estimated by a modification of the arginase-urease method of Edlbacher and Röthler [1925]. In accordance with the procedure outlined above for the examination of a suspected enzyme, a highly potent glycerol solution of arginase was prepared from fresh liver [Edlbacher and Röthler, 1925]. The amounts of this arginase solution and of the B.D.H. trypsin preparation were then calculated from their arginase activities, so that, if arginase were the inactivating enzyme, the arginase solution should have caused approximately twice as much inactivation of the hormone as the trypsin preparation—twice as much, rather than the same, inactivation,

[1] This term denotes the well-defined enzyme with known substrate which is suspected of attacking the hormone. The term "inactivating enzyme" denotes the unknown enzyme in a preparation which attacks the hormone.

in order to emphasise the result. When these enzymes were examined under identical conditions for their action on the hormone, the trypsin preparation reduced the activity to 7 % of its initial value in 30 minutes, whereas with the liver extract no inactivation was detectable after 4 hours. Thus the inactivating enzyme of the B.D.H. trypsin preparation is not arginase. The papain preparation used in the earlier experiments exhibited no arginase activity, and hence the hormone does not contain the group I, although the group II or another guanidine derivative is not excluded [Felix *et al.*, 1928; Edlbacher and Burchard, 1931].

The shape and position of the optimum of the p_H-activity curve of the inactivating enzyme of the yeast and papain preparations (Part III) closely resembled those (Fig. 3) of prolinase, of which the simplest substrate is prolylglycine [Grassmann *et al.*, 1932]; the substrates of this enzyme all contain the

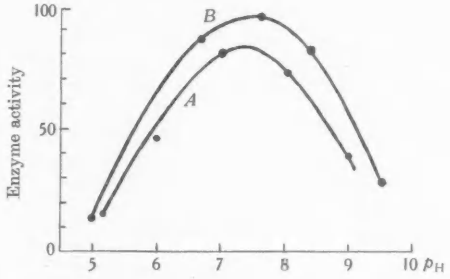

Fig. 3. p_H-activity curves of inactivating enzyme of yeast proteinase solutions (*A*) and of Grassmann's prolinase (*B*).

basic imino-group of proline. When the yeast dipeptidase, yeast proteinase and papain preparations used in Part III were tested for prolinase activity, all contained traces of this enzyme. It seemed probable that these amounts were too small for prolinase to be the inactivator, but, on the other hand, the affinity of the inactivating enzyme for the hormone is unknown and may be very great. The routine examination already described was therefore undertaken. A solution of prolinase, prepared from a neutral autolysate of yeast, contained more than 50 times the prolinase activity of the yeast proteinase solution. From this figure, a calculation was made of the amounts of the two enzymes needed to compare their power of inactivating the hormone; as before, however, only half the requisite amount of proteinase preparation was used, in order to emphasise the result. The enzyme of the proteinase solution reduced the oxytocic value to 38 % in 30 minutes, whereas with the yeast autolysate it had only become 90 % in that time, and after 4 hours was still 73 %. Thus, the inactivating enzyme of the yeast and papain preparations is not prolinase.

The B.D.H. trypsin preparation contained moderate amounts of an enzyme which hydrolysed prolylglycine. The dependence of the activity of this enzyme

on hydrogen ion concentration resembled that of prolinase [Grassmann *et al.*, 1932] in having an optimum just below p_H 8. It was therefore improbable that the inactivating enzyme (optimum at p_H 8·8) of the B. D. H. trypsin preparation was prolinase.

Experimental.

As in previous communications, the oxytocic activity of solutions was assayed by the degree of contraction they produced in the isolated uterus of the guinea-pig. The technique described in Part III was adopted in the present experiments.

Action of B. D. H. trypsin preparation at p_H 7·5.

A hormone solution (2·0 cc., containing 156 oxytocic units per cc.), *M*/3 sodium phosphate at p_H 7·5 (3 cc.) and a solution of B. D. H. trypsin preparation (10 mg. in 2 cc. of water) were mixed, and the mixture diluted to 10·0 cc. at p_H 7·5 by means of water and a trace of *N* ammonia. A control sample (1·0 cc.) was immediately removed into a 2·5 cc. flask, heated for 3 minutes in a boiling water-bath and placed in a thermostat at 40°. The remainder of the mixture had meantime been placed in the thermostat. Further samples were removed at appropriate intervals, and finally all were diluted to 2·5 cc. at p_H 3·5 with 2·5*N* acetic acid (0·5 cc.) and water. Each sample was assayed against the control solution, 0·2 cc. being diluted to 10 cc. at p_H 7·5; these dilutions would therefore have contained 0·25 unit per cc. if no inactivation had occurred. The results are given in Table I.

Table I.

Time (hours)	0·5	1	6	27
Activity present (%)	0·7	0·5	0·3	0·3

Action of Kahlbaum's trypsin at p_H 7·5.

The amounts of hormone, enzyme and buffer were those of the preceding experiment, and the procedure was exactly the same (Table II).

Table II.

Time (hours)	1	6	24
Activity present (%)	100	76	40

Comparison of the action of B. D. H. and Kahlbaum trypsin preparations.

A. *Proteolytic activity.* The proteolytic activities of the two preparations were compared by the method of titration in 90 % alcohol with alkali, gelatin being used as substrate. Gelatin solution (6·0 cc. of 10 %), *M*/3 sodium phosphate buffer at p_H 7·5 (1·0 cc.) and varied amounts of the solid enzyme preparations were mixed and diluted to 10·0 cc. at p_H 7·5, the p_H being adjusted with ammonia or acetic acid. In each case a sample (1·0 cc.) was immediately removed into 9 cc. of absolute alcohol at 60°, this mixture being titrated with *N*/20 alcoholic KOH whilst being shaken. The reaction mixtures were incubated at 40°, and further samples were taken at the times stated in Table III.

It is evident (see especially columns marked †) that the proteinase activity of the B. D. H. preparation was about 50 times greater than that of the Kahlbaum preparation. By comparing only the increases in acidity in the early stages of the hydrolysis, the effects due to ereptic action may be disregarded or estimated. Of the two preparations, the B. D. H. one is richer in ereptic activity; this is shown by the greater increase in acidity in the later stages of the experiments in which it is used.

Table III.

The figures denote increases in acidity of 1 cc. portions of the reaction mixtures, expressed as cc. of $N/20$ KOH. The weight of the enzyme preparation used is shown above each column.

Kahlbaum trypsin prep. (Dec. 1922)				Time	B.D.H. trypsin prep. (May 1931)			
10 mg.*	20 mg.	100 mg.	500 mg.†	hours	1 mg.	2 mg.	10 mg.	10 mg.†
—	—	—	0·38	¼	—	—	—	0·38
—	—	—	0·68	½	—	—	0·65	0·74
—	—	0·38	0·76	1	0·23	0·45	—	0·96
0·05	—	0·46	—	2	0·40	0·53	—	—
—	—	—	—	5	—	—	1·14	—
0·15	—	—	—	5½	—	—	—	—
—	—	0·60	—	6	0·82	0·88	—	—
—	0·47	—	—	17	—	—	—	—
—	—	—	—	20	—	—	1·48	—
0·52	—	—	—	23	—	—	—	—
—	0·51	—	—	41	—	—	—	—
0·60	—	—	—	47	—	—	—	—

* The reaction mixture contained 1·0 cc. of $N/2·5$ ammonium chloride in addition to the other components.

B. *Inactivation of the hormone.* The experiments with both enzymes were made under identical conditions. A hormone solution (1·0 cc., containing 156 units), $M/3$ sodium phosphate buffer at p_H 7·5 (1·5 cc.) and a solution of the enzyme preparation (125 mg. of Kahlbaum trypsin, or 2·5 mg. of B.D.H. trypsin) in water (0·5 cc.) were mixed, adjusted to p_H 7·5 with N ammonia and diluted to 5·0 cc. with water. The customary procedure was observed, the samples measuring 1·0 cc. and being taken at the same intervals. The samples were assayed against their control samples, 0·2 cc. in each case being diluted to 10 cc. at p_H 7·5. The results are given in Table IV and Fig. 2.

Table IV.

Kahlbaum prep., activity present (%)	Time hours	B.D.H. prep., activity present (%)
86	½	19
71	1	2·7
59	2	0·6

These results showed that the inactivation of the hormone was not caused by trypsin, assuming that the different rates of inactivation were not due to a more rapid destruction of the trypsin in one preparation than in the other. It was shown that if destruction did occur, it was approximately equal in the case of both enzymes. At the end of the incubations the residual proteinase activity was determined as follows. Each enzyme-hormone-buffer mixture (1 cc.) was mixed with 10 % gelatin solution (3 cc.), and the mixture was adjusted to p_H 7·5, diluted to 5 cc. with water and incubated at 40°. The rate of liquefaction of the gelatin was then followed. After 1 hour the gelatin of both mixtures solidified with great difficulty when cooled under the tap, the times required for solidification being approximately the same. After 2 hours the gelatin of both experiments did not solidify.

Effect of hydrogen ion concentration on the rate of inactivation of the hormone by the B.D.H. trypsin preparation.

An aqueous solution of B.D.H. trypsin (1·25 mg. in 0·25 cc.) was added to an ice-cold mixture of a hormone solution (0·5 cc., containing 156 units per cc.) and *M*/3 sodium phosphate buffer at the required p_H (1·75 cc.). A control sample was immediately removed into a 2·5 cc. flask, heated for 3 minutes at 100° and placed in the thermostat at 40°. The remainder of the solution was kept in the thermostat for 30 minutes, when a second sample was taken as described above. The p_H of the remainder of the solution was determined and taken as the p_H of the experiment; determinations at p_H 9·3 and 10·2 were made with a hydrogen electrode, the others colorimetrically.

The experimental solutions were assayed against the control solutions, 0·2 cc. in each case being diluted to 10 cc. at p_H 7·5. The results are given in Table V and Fig. 1.

Table V.

p_H	5·2	6·0	7·0	7·5	8·0	8·8	9·3	10·2
Inactivation (%)	0	26	67	82·5	90	95	92·5	81

Action of B.D.H. pepsin preparation.

A. *At* p_H *7·2.* A hormone solution (6·7 cc., containing 3 units per cc.), *M*/3 sodium phosphate solution at p_H 7·5 (1 cc.) and a solution of active B.D.H. pepsin (100 mg. in 2 cc. of water) were mixed and diluted to 10 cc. at p_H 7·2. A control sample (2·0 cc.) was immediately removed, and other samples were taken at ½, 2, and 25 hours. The usual technique was followed.

B. *At* p_H *4·4.* The procedure was exactly as described above, except that *N* acetate buffer was used. The p_H of the mixture was 4·4. In both series of experiments the samples were assayed against the controls, 1 cc. being diluted to 10 cc. In no case could any inactivation be detected.

Action of bone phosphatase. A hormone solution (0·25 cc., containing 312 units per cc.), *M*/5 sodium phosphate at p_H 7·8 (1 cc.) and a solution of a bone phosphatase preparation (10 mg. of A/W = 0·23 [Martland and Robison, 1929]) in water (1 cc.) were mixed and diluted to 5 cc. with water. The p_H was 7·8. The usual technique was followed, samples (1 cc.) being removed at ½, 1½, and 26 hours and assayed against the control sample. No inactivation of the hormone was detected.

Consideration of the possibility that arginase inactivates the hormone.

A. *Estimation of arginase in B.D.H. trypsin preparation.* A solution of trypsin (100 mg.) in water (2 cc.), *M*/10 glycine-sodium hydroxide buffer at p_H 9·5 (2 cc.) and a 1 % *d*-arginine solution (2 cc.) were mixed, and the mixture was adjusted to p_H 9·5, incubated for 1 hour at 40° and heated for 4 minutes at 100°. The p_H of the mixture was then adjusted to 7, and a suspension of one powdered tablet of B.D.H. urease[1] in *M*/3 phosphate buffer at p_H 7 (1 cc.) was added. The mixture, in a closed tube, was incubated at 40° for 1 hour, cooled and transferred to a micro-Kjeldahl apparatus containing saturated sodium carbonate solution (2 cc.) and some medicinal paraffin (to prevent frothing). Steam was passed into the apparatus in the usual way[2], and the

[1] One tablet hydrolyses 50 mg. urea in 1 hour at 35°.

[2] This is a modification of the method of Edlbacher and Röthler [1925]. They aspirated the ammonia from the warm solution, but we obtained inconsistent results owing to incomplete removal of ammonia.

distillate was collected in $N/50$ hydrochloric acid (10·0 cc.); after actual distillation had proceeded for 3 minutes, the contents of the receiver were titrated to methyl red with $N/50$ NaOH. Control experiments were also carried out following the same procedure, except that the trypsin-buffer-arginase mixture was heated for 4 minutes before incubation instead of afterwards. The experiments were repeated several times, and in all cases a small but definite arginase activity was detected. It was estimated that under the conditions of the experiment 100 mg. of the trypsin preparation yielded 0·10–0·15 cc. of $N/50$ ammonia.

B. *Estimation of arginase activity in a preparation from liver.* The arginase content of a glycerol extract [Edlbacher and Röthler, 1925] of guinea-pig liver was estimated as described above. The 1:3 glycerol extract (1 cc.) was diluted to 10 cc., and 0·2 and 0·4 cc. of the dilution used for the estimation (Table VI).

Table VI.

Diluted glycerol extract (cc.)	Experiment cc. $N/50$ NaOH	Control cc. $N/50$ NaOH	Ammonia formed cc. $N/50$
0·2	7·48	9·69	2·21
0·4	6·08	9·65	3·57

C. *Comparison of hormonal inactivation by B.D.H. trypsin and arginase preparations.* The two experiments followed the customary procedure of assaying experimental against control solutions and need not be described in detail. The volumes of the reaction solutions were 2·5 cc., the p_H values were 9·3, and the amounts of hormone used were comparable. In one experiment the B.D.H. trypsin preparation (1·25 mg.) was used, in the other a solution containing 0·00004 cc. of the glycerol extract of guinea-pig liver. This solution thus contained about twice as much arginase activity as the amount of trypsin preparation used. The trypsin preparation effected 92·5 % of the possible inactivation in ½ hour, whereas little or no inactivation (possibly as much as 10 %) occurred after 4 hours in the experiment with arginase solution. If arginase inactivated the hormone, the solution of the glycerol extract of arginase should have caused about twice as much inactivation as the trypsin preparation. It follows, therefore, that the inactivating enzyme of the trypsin preparation was not arginase.

The B.D.H. papain preparation contained no arginase, nor did it exhibit any urease activity, which would have interfered with the arginase estimation. The inactivating enzyme of yeast and papain preparations is not arginase.

Consideration of the possibility that prolinase inactivates the hormone.

A. *Estimation of prolinase in preparations of yeast proteolytic enzymes.* Prolinase activity was measured by titrating the increased acidity of prolylglycine with alkali in 90 % alcohol [Grassmann *et al.*, 1932]. *dl*-Prolylglycine (40 mg.) was dissolved in $M/3$ phosphate buffer at p_H 7·5 (0·25 cc.) in a 2·5 cc. flask. An aqueous solution (1·5 cc.) of a yeast dipeptidase, yeast proteinase or B.D.H. papain preparation was added, and the mixture was diluted with water and alkali to 2·5 cc. at p_H 7·5. The enzyme preparations were those used in Part III. A sample (0·5 cc.) was immediately removed into a flask containing absolute alcohol (4·5 cc.) and titrated to thymolphthalein with $N/20$ potassium hydroxide in 90 % alcohol. The remainder of the solution was incubated at 40°, and samples were taken at 4 and 23 hours. In the control experiment the enzyme solution was replaced by water. The results are given in Table VII, and it will be seen that each enzyme preparation contains traces of prolinase.

Table VII.

The figures represent the degree of hydrolysis and are the increase in acidity of 0·5 cc. samples quoted as cc. $N/20$ alcoholic KOH.

Time hours	Control	Dipeptidase 5 mg.	Papain 5 mg.	Proteinase 0·5 cc.
4	0·00	0·01	0·02	0·01
23	0·00	0·06	0·04	0·02

B. *Preparation of a potent prolinase solution from yeast.* English brewer's yeast (50 g.) was liquefied with ethyl acetate (5 cc.) and allowed to autolyse for 1·5 hours at p_H 6·5–7, ammonia being added to maintain the p_H. The yeast was centrifuged, washed and then allowed to autolyse at p_H 7·0 for 24 hours in presence of a few drops of toluene. Finally the yeast was centrifuged, and the prolinase activity of the clear solution was estimated as described above. Acting on *dl*-prolylglycine (40 mg.) for 2 hours at p_H 7·5 and 40° in a total volume of 2·5 cc., the prolinase solution (0·5 cc.) caused an increase in the acidity of 1·0 cc. portions corresponding to 0·24 cc. of $N/20$ KOH. The prolinase solution had therefore more than 50 times the prolinase activity of the proteinase solution (above).

C. *Comparison of hormonal inactivation by yeast proteinase and prolinase solutions.* Hormone solutions (each containing 30 units) were mixed either with the proteinase solution (0·25 cc.) or with an aqueous dilution of the yeast autolysate corresponding with 0·007 cc. of this autolysate; the experiment with the proteinase thus contained about half the prolinase activity of that with the yeast autolysate. The mixtures were diluted to 2·5 cc. at p_H 7·0, being buffered with $M/15$ sodium phosphate, and were incubated at 40°. The usual procedure was followed, samples being removed immediately and after 30 minutes; a sample from the yeast autolysate experiment was also taken after 4 hours. In the proteinase experiment 38 % of the initial oxytocic activity remained after 30 minutes, whereas in the yeast autolysate experiment more than 90 % was present after that period, and after 4 hours 73 % still remained. The inactivating enzyme of the yeast and papain preparations is therefore not prolinase.

D. *Action of B.D.H. trypsin preparation on prolylglycine. dl*-Prolylglycine (40 mg.) in $M/3$ phosphate buffer at the appropriate reaction (0·25 cc.) was mixed with a solution of B.D.H. trypsin preparation (25 mg.) in water (0·5 cc.) in a 2·5 cc. flask. The mixture was adjusted to the required p_H and diluted to 2·5 cc. Samples (1·0 cc.) were removed for titration in the usual way at the beginning and after 2½ hours. The control experiments contained no substrate. The results are given in Table VIII.

Table VIII.

The figures represent increases in acidity in terms of $N/20$ KOH.

p_H	6·0	7·0	8·0	9·0
Experiment	0·16	0·27	0·44	0·29
Control	0·06	0·07	0·12	0·16
Due to prolinase	0·10	0·20	0·32	0·13

Difficulties encountered in determining the end-points increased the experimental error of these results. It is evident however that the p_H optimum of the prolinase is considerably less alkaline than p_H 9 and probably less than p_H 8. The inactivating enzyme of the trypsin preparation, having an optimum at p_H 8·8, is probably not prolinase.

An experiment similar to that described above was carried out with the Kahlbaum trypsin preparation (125 mg. in 2·5 cc.) at p_H 7·2. No increase in acidity was observed after incubation for 2·5 hours. This preparation, therefore, did not contain prolinase.

SUMMARY.

1. A B.D.H. trypsin preparation inactivated the hormone very rapidly at p_H 7·5, the p_H optimum of the inactivation being 8·8.

2. Under similar conditions a Kahlbaum trypsin preparation inactivated the hormone extremely slowly.

3. Experiments in which quantities of these preparations having equal tryptic activities acted on the hormone showed that, contrary to existing conclusions, the inactivating enzyme was not trypsin. This result, conjoined to the earlier proof that the inactivating enzyme of papain preparations is not a papainase, affords strong evidence against the contention that the hormone is proteinoid.

4. In attempts to identify the inactivating enzyme of yeast and papain preparations and also that of the B.D.H. trypsin preparation, it was shown that neither arginase nor prolinase inactivates the hormone.

5. No evidence is yet available to support the view that the hormone contains peptide linkages.

6. The hormone was not attacked by a B.D.H. pepsin preparation at p_H 4·4 or at p_H 7·2, or by a bone phosphatase preparation at p_H 7·8.

Our thanks are due to Messrs Boots Pure Drug Company, Nottingham, who generously presented the posterior lobe powder used in this work.

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CLXXXVIII. THE QUANTITATIVE EXTRACTION OF HISTAMINE FROM TISSUES BY ELECTRODIALYSIS.

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During an investigation involving a large number of histamine assays, it occurred to us that the chemical manipulation could be greatly simplified if the base could be extracted by electrodialysis. A further advantage of the method would be the elimination of all but basic substances of low molecular weight from the extract. In the method we had been using [Best *et al.*, 1927] the tissue is extracted with 96 % alcohol for 24 hours, the alcohol removed and the meat re-extracted with 60 % alcohol for a further 24 hours. The combined extracts are concentrated *in vacuo* and fat removed by extraction with ether. The extracts so prepared were usually yellow and often slightly cloudy, although free from biuret products.

By electrodialysis in a three compartment cell a water-clear colourless extract is obtained in less than 2 hours after receipt of the tissue: this means a great saving of time when many samples have to be worked up. In a large number of experiments during the last 3 years we have never found an electrodialysate giving a smaller histamine value than an equivalent dose of an extract prepared by the alcohol method. (The histamine value of the extracts was obtained by matching against a standard of pure histamine on the blood pressure of an anaesthetised cat.) Further, histamine added to blood, which contains no histamine, has been repeatedly extracted without loss. Histamine added to tissues which contain histamine has also been recovered without detectable loss.

The process of electrodialysis does not subject the tissues in the middle compartment to any drastic conditions. The temperature does not rise above 40°, and there is no great change in the p_H of the liquid in the middle compartment. The method therefore supports the alcohol method not only quantitatively but also in the suggestion that the histamine in the tissues is, if not free, very loosely combined.

On a few occasions we have also extracted a portion of the tissue by the more drastic method of Best and McHenry [1930], in which the tissue is heated with 10 % HCl at 95° for 1 hour and then evaporated to dryness *in vacuo*; alcohol is added and distilled off to remove some of the acid, and the residue is taken up in water, neutralised and filtered. These extracts, which are clear but dark brown in colour, usually give a value about 30 % higher than extracts prepared by either of the above methods. In view of the agreement between the alcohol and dialysis methods and our quantitative recovery of added histamine, we feel that it is highly significant that extracts prepared by the Best and McHenry method give a strong biuret and several other protein reactions

not given by the alcohol extracts or electrodialysates of the same tissue; especially since we have observed an increased histamine value when a membrane passed biuret products.

It might be argued that the lower value obtained by the dialysis is due to loss of histamine by autolysis [Best, 1929] in the dialysis cell, whilst the lower value by the alcohol method is due to incomplete extraction. Apart from the coincidence of the errors, it should be noted that the time for which the tissue is in the region of 37° rarely exceeds half an hour. This short time would not be sufficient, judging from Best's curve, to cause any perceptible loss in histamine. Again, Best *et al.* [1927] were unable to detect any difference in the histamine content of a dog's lungs worked up immediately after removal from the animal or after keeping 2 hours; and MacGregor and Peat [1931] found no change in the histamine in a lung mince which had stood overnight.

Experimental.

Description of apparatus.

Cells. The cells used for these experiments were, with one exception, made from wood to the pattern of those of Foster and Schmidt [1923]. Small cells of 100 cc. capacity in each compartment were carved from solid blocks of well-seasoned beech or Oregon pine. Larger cells of 250 cc. capacity (Foster and Schmidt size) were built up from pieces of thick wood. The wood was well impregnated with high-melting paraffin wax. The waxing is of some importance since a badly waxed cell often gives up during dialysis an alkali-soluble substance giving a strong Pauly reaction. This substance is insoluble in neutral or acid solution. Still larger cells used in some of the preliminary experiments were made from three glass museum jars, faces $7'' \times 5\frac{1}{2}''$, with $3\frac{1}{2}''$ holes cut in the appropriate faces. The capacity of each compartment varied from 600 cc. to 1500 cc. according to the width of the jars used. For the wooden cells the gaskets were made of ordinary red rubber sheet $\frac{1}{16}''$ thick. Owing to their fragility the glass cells required special soft rubber gaskets $\frac{3}{8}''$ thick. These were specially made for us by Messrs C. Macintosh and Co., Ltd., Manchester.

Electrodes. The cathodes were made from pure nickel sheet slightly smaller than the cross section of the cell. The anodes were made from one or two $6'' \times 2'' \times \frac{1}{4}''$ carbon plates according to the size of the cell.

Membranes. In the experiments with lung and blood ordinary parchment dialysing-paper or cellophane sheets were found quite satisfactory. With liver and heart, especially the former, these membranes passed traces of "biuret" products, and the dialysates gave histamine values perceptibly higher than those obtained from the corresponding alcohol extracts. The passage of such substances is prevented by using cathode membranes of collodion. We found it simpler to make sacs by the inside tube method in $10'' \times 1\frac{1}{2}''$ test-tubes than to make sheets direct by the plate or mercury method. The sacs were then cut up into sheets and kept in distilled water until required. 14 % pyroxylin in equal parts (by weight) of ether and absolute alcohol [Walpole, 1915] gave sufficiently robust membranes.

Stirring. The liquid in the middle compartment was kept stirred mechanically to prevent any local aggregation of mince. Some tissues, especially heart muscle, tend to form a layer on the cathode membrane which increases somewhat the electrical resistance of the cell and thus slightly increases the time required for dialysis. This layer was broken up from time to time by a glass rod.

Current. The cell was connected to 230 volt D.C. mains in series with a resistance of a bank of 4 carbon filament lamps in parallel. Each lamp passed about 1 amp. and could be switched into or out of the circuit as required. For the smaller cells 2 or 3 lamps were generally sufficient. The mean consumption of the 100 cc. cells was about 50 watt-hours, and of the 250 cc. cell about 120 watt-hours.

Temperature. The temperature was kept down by providing each compartment with glass cooling coils supplied with a rapid stream of cold water. In this way the temperature even in the smallest cell could be kept below 40° when the current passing was 1 amp. The temperature only reached this value while a heavy current was passing. This rarely occurred for more than half an hour. A typical curve showing the current and temperature changes is given in Fig. 1.

p_H. The p_H change in the middle compartment was not sufficient to warrant alteration, *e.g.* by addition of baryta. With tissues, the p_H in the middle compartment, which is initially just above 7, usually falls to about 4·6. We have never observed a p_H more acid than this. With blood, the p_H does not usually fall below 6.

End-point. Theoretically, when the resistance of the cell has attained a steady value and the titratable alkalinity is constant, all the histamine should have passed to the cathode chamber. In view, however, of the difficulty of accurate current measurement without special instruments and the minute amount of histamine present relative to other electrolytes, we found it more reliable to use the Pauly reaction as an indication of the completion of the dialysis. Samples were withdrawn from the cathode chamber at 15 minute intervals until two successive samples gave the same colour. For this purpose we have adapted Gebauer-Fülnegg's [1930] modification of the reaction as a quantitative method. 1 cc. of test solution is mixed with 1 cc. $N/2$ Na_2CO_3 and to this are added 2 cc. of fresh diazo-reagent prepared by mixing 1 cc. of 0·125 % *p*-nitraniline in $N/10$ HCl with 1 cc. 0·37 % $NaNO_2$. The colour gradually deepens and then fades, finally becoming cloudy. The maximum colour intensity, which usually appears in about 1 minute, is recorded in red and yellow units in a Rosenheim and Schuster [1927] colorimeter. Concentrations from 1/33,000 to 1/300,000 can be read off directly without dilution. Plotting colorimeter red units against histamine concentration gives an approximate straight line over this range. The method gives consistent results and for our purpose is quicker and more convenient than the Koessler and Hanke [1919] method which we used in our early experiments. But it must be stressed that this, like other adaptations of the Pauly reaction, is not specific for histamine and cannot be used for the estimation of the base in tissue extracts. In every case our Pauly values have been higher than would be expected from the blood pressure assay. The reaction could be used as an indicator for the extraction from tissues of any alkali-stable base which passes to the cathode with the same ease as histamine.

Since the convenience of this end-point depends upon the use of a Rosenheim and Schuster colorimeter, we have studied the current relations throughout the experiments with a view to giving an alternative method for determination of the end-point. Measurements of the voltage across the cell and amperage of the circuit were noted every 15 minutes. From the area of a watt-time curve drawn from these data the total consumption in watts was obtained. In 60 experiments with 4 different cells we have found the ratio WT/ad to be approximately constant for a given tissue. T is the time taken to attain a constant

Pauly value, a the area of the cathode in cm. and d the distance in cm. of the cathode from the centre of the middle compartment. The mean values of this ratio are given in Table I. (In only 5 cases were the values more than $\pm 0{\cdot}1$ from the mean.) As would be expected these values are roughly proportional to the mean values for the titratable alkalinity of the cathode liquids. The latter values, expressed as cc. N acid per 100 cc. of dialysate, and the weight of tissue corresponding to this volume are also included in the table.

Table I.

Tissue	$\frac{WT}{ad}$	Titratable alkalinity cc. N acid	Weight of tissue g.	Number of experiments
Blood	0·63	3·9	30	15
Heart-muscle	0·49*	2·6	20	18
Liver	0·43	2·6	20	11
Lung	0·53	3·3	20	16

* This value is probably relatively high, since with heart-muscle there was a much greater accumulation of tissue on the cathode membrane than with any other tissues examined.

Since we had no indication in any experiment that prolonging the dialysis caused any loss of histamine, it should be safe to assume the complete passage of any equally stable and dialysable base when sufficient current has been passed to satisfy the equation $WT/ad = 0{\cdot}6$ for the tissues or $WT/ad = 0{\cdot}7$ for blood.

Method.

The cell having been assembled, the cathode and anode compartments are filled to capacity, say 250 cc., with distilled water. The middle compartment is filled with 200 cc. distilled water and 50 g. of the minced tissue. It is not necessary to add an electrolyte to the distilled water. After turning on the cooling water and starting the stirrer, the current is switched on. If the small cells are used, the current and temperature in the middle compartment, which rise rapidly at first, fall after about 1 hour. The end-point is then determined as already described (p. 1396). The cathode liquid is then removed and the chamber washed out. The combined liquid and washings after neutralisation with N H_2SO_4 and adjustment to appropriate volume are ready for the physiological assay.

Physiological assay. The extracts were compared as to their depressor effect when injected into the femoral vein of an anaesthetised cat. At least two definite matches were obtained before the pairs of extracts were recorded as equal. Histamine values were obtained by matching against a standard histamine solution [Best *et al.*, 1927; Burn, 1928]. The cats were anaesthetised with medinal (0·42 g. per kg. body weight) after induction with ether.

Results.

Comparison of alcohol and electrodialysis methods. Three histamine-containing tissues were examined. In all the experiments the tissue was minced and well mixed. Samples were then worked up by the dialysis method and the alcohol method and assayed physiologically. There was no detectable difference. These experiments are summarised in Table II, which also shows the extreme histamine values for each tissue.

Table II.

Tissue	Source	Number of experiments	Extreme histamine values in mg. per kg.
Heart (ventricular muscle)	Ox	8	8–25
Liver	Dog	2	13–40
	Ox	2	10–20
	Pig	2	13
	Sheep	3	2·5–6
Lung	Dog	3	10–13
	Ox	11	30–60

In the case of the liver which contains choline, the dialysates and the alcohol extracts were equidepressor both before and after injection of atropine into the cat, indicating, as would be expected, that choline passes to the cathode like histamine.

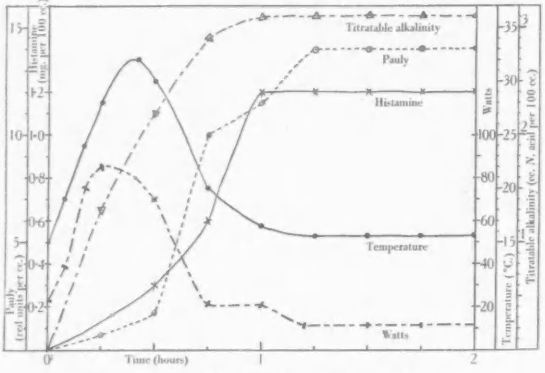

Fig. 1. Electrodialysis of 20 g. lung in 100 cc. cell.

× ——— × Histamine values of cathode liquid.
⊙ – – – ⊙ Pauly values of cathode liquid.
△ – – – △ Titratable alkalinity values of cathode liquid.
● ——— ● Temperature of mixture in centre compartment.
\+ – – – + Watt values.

Reliability of end-point. In nine experiments samples were withdrawn at 15 minute intervals, neutralised and injected into an anaesthetised cat. The results from one of these experiments are shown in Fig. 1. Similar curves were obtained in the other experiments. They showed that:

1. The Pauly value never attained a maximum before the histamine concentration.

2. The titratable alkalinity value usually reached a maximum before the histamine concentration.

3. The current usually but not always reached a minimum after the histamine concentration became maximum.

Thus the Pauly reaction was the only reliable method of the three for determining the end-point.

Recovery of added histamine.

1. *From histamine-free material.* A known volume of histamine solution was added to a 30 % blood solution and electrodialysed. The same volume of histamine was added to an appropriate volume of water. The dialysate was then

concentrated *in vacuo* to this volume, and the two solutions were sterilised and preserved until compared on the cat's blood pressure. The amounts of histamine added were such as to give a similar concentration in the cell to that given when a tissue was being dialysed, *i.e.* 2, 1 or 0·5 mg. were added to the 250 cc. cell and 0·8, 0·4 or 0·2 mg. to the 100 cc. cell. These amounts correspond to tissues containing 40, 20 and 10 mg./kg. respectively. In nine experiments the base was recovered quantitatively so far as could be detected by the physiological assay. It has already been stated in a preliminary communication [MacGregor and Thorpe, 1933] that a large amount of histamine (0·06 g.) added to blood was easily recovered in the form of its picrate in good yield.

2. *From tissues containing histamine.* Failure to recover completely histamine which has been added to tissues has often been reported. These experiments have been carried out by adding histamine to one portion of the tissue and working up another portion without added histamine. The difference between the two extracts is then estimated by matching them against a standard histamine solution on the cat's blood pressure, and hence the amount of added histamine is calculated. The method, especially when the differences are great, is liable to big errors owing to the large multiples involved in the calculations. In our experience the stronger solutions tend to be underestimated. Being of the opinion that this was largely responsible for the failures to recover added histamine, we planned our experiments so as to use equipotent solutions and to eliminate all calculations.

A known volume of a histamine solution was added to a portion of the tissue and the mixture dialysed. An equal portion of the same tissue was dialysed in another cell and the same volume of histamine added to the dialysate after removal from the cell. The two dialysates were then neutralised, adjusted to the same volumes and equal doses compared on the cat's blood pressure. In five experiments no difference could be detected between the two solutions. The histamine had been recovered quantitatively within the limits of the physiological assay.

SUMMARY.

1. A rapid method for the quantitative extraction of histamine from tissues by electrodialysis is described.

2. The method gives results in complete agreement with the alcohol method.

3. Added histamine is recovered quantitatively within the limits of the physiological assay.

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CLXXXIX. A STUDY OF THE COMPOSITION OF HUMAN MILK.

THE INFLUENCE OF THE METHOD OF EXTRACTION ON THE FAT PERCENTAGE.

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The fat content of both cow's and human milk is a subject on which much work has been done, and though certain facts are now well established there is still no clear understanding of the circumstances which determine the normal range of variation or knowledge of the conditions which determine these comparatively wide limits. In the course of our study on the composition of human milk in the early stages of lactation [Lowenfeld *et al.*, 1927; 1928] it was noticed that the method of extraction of the milk sample had a considerable influence on the percentage of fat, although no other constituent was affected by this factor. Since this factor had not to our knowledge been investigated by any previous worker, it occurred to us that herein might lie the probable explanation of the variations in the fat percentage hitherto recorded. We decided therefore to undertake a more intensive study of the fat content in human milk throughout the whole course of lactation, paying special attention to the method adopted for obtaining the milk sample. The present paper is a record of our results and the conclusions which they suggest.

Methods of obtaining milk.

The two methods used for obtaining the samples of milk (for convenience called the "extraction" of milk) throughout the work were either digital expression or gentle use of the breast pump, and in every case where milk dripped from the breast a sample of this milk was also collected in addition to the other samples.

These two methods were adopted after critical observation of the actual process of suckling by an infant. This process consists of two factors, the direct pressure exerted by the gums of the baby upon the nipple and the areola of the breast, and the suction action exercised by tongue and cheek drawing on the openings at the end of the nipple. Digital pressure and gentle suction from a pump used without pressure upon the breast represent the nearest approach which can be made artificially to reproduction of these two factors in the normal process. Systematic comparison made of the percentages of fat in samples of milk extracted by these two methods, under similar conditions, at once showed very marked differences. Indeed, the differences were so striking that we have come to the conclusion that in the method of extraction is to be found the chief explanation of the wide variations shown in fat figures obtained by different workers.

Material.

Twenty hospital cases were used for investigation of the milk of the first fortnight of lactation, and a continuous study of the milk of the same mother throughout this period was thus possible.

Owing to irregularities in attendance at hospital or clinic, it was not possible to achieve the same continuity in the later period of lactation. Nevertheless, every effort was made to examine the milk of the same woman over as many months as possible. By a combination of the figures obtained from the two sets of investigations, material sufficient for a general survey of the problem of the fat percentage in milk under varying conditions and during the whole course of lactation has been obtained.

The milk of 51 mothers was examined for the percentage of fat, protein, sugar, calcium, phosphorus and ash, and the results of this work, except for that on fat, have already been recorded [Lowenfeld *et al.*, 1927; Widdows *et al.*, 1930]. The milk samples were obtained from nursing mothers in the lying-in wards of the Obstetric Unit of the Royal Free Hospital, from those attending the Shoreditch Carnegie Welfare Settlement, and the Mothercraft Training Society, Cromwell House, Highgate.

Careful notes were taken of the mother and child so that all abnormal factors could be excluded. In every instance, except for ten cases, the baby was thriving satisfactorily. In these ten cases an attempt was made to determine the cause of the nutritional disturbance in the infants.

To maintain uniformity of conditions the milk was always taken for analysis at the same feeding time, and the same interval was observed between the times of feeding. The importance of these factors had been shown in the case of cows by Eckles and Shaw [1913] who state that the percentage of fat is usually highest about midday, and in a Bulletin recently issued by the Ministry of Agriculture and Fisheries [1931] instances are given from the work of Gilchrist [1925] and Mackintosh [1925] fully confirming this diurnal variation. In the case of human milk, Helbich [1911] showed the highest percentage to be at 10 a.m. or 2 p.m., and more recently Deem [1931] has again emphasised this, showing that the percentage of fat is at a maximum at 10 a.m., nearly the same at 2 p.m., and at a minimum at 6 a.m.

In our work all the samples in the early days of lactation were taken at 10.30 a.m. and during the later periods at 2 p.m.

The taking of the sample, except in the case of the Mothercraft Training Society, was carried out by one of us (M. F. L.) and the biochemical analysis by the other (S. T. W.).

Every visit of each mother necessitated, as will appear later, at least four estimations of fat, and where milk dripped from the breast a sample of this dripped milk was also taken for estimation. In all, 276 samples taken before and after the baby had fed and 18 dripped samples were collected and analysed.

Analytical methods.

Gottlieb's method was used for the estimation of the fat.

To study the influence of the method of extraction on the fat content of the milk 216 samples were examined. These were obtained according to the following procedure.

From one breast a sample sufficient for analysis was taken by hand, the amount varying from 2 to 30 cc. according to the average yield of the subject.

The baby was then allowed to feed from 2 to 5 minutes, and the breast was then finally emptied by hand, the total resulting fluid being considered as the second sample.

From the other breast also a sample was taken before feeding, by hand; the baby was allowed to feed for the same length of time as before, and the final sample, which emptied the breast, was obtained by gentle use of the pump, avoiding pressure upon the breast.

The use of the two different methods, keeping the other conditions constant, enabled the bearing of each upon the fat percentage to be studied. On the one side a uniform method of extraction was employed, namely digital expression, for both the samples. On the other side digital expression was used in obtaining the first sample and suction by the breast pump for the last sample.

Our previous work has shown that in normal cases the composition of the milk given by either breast is approximately the same. Assuming this to be generally the case in all women, if the manner of extraction were immaterial, even although the exact figures of the percentage might differ on either side, a rise in the fat percentage on one side would be paralleled by a similar degree of rise on the other. It is therefore the question of the relation of the method of extraction to the production of the degree of rise in the fat percentage between the beginning and end of a feed with which we are at present concerned.

Before considering our results, certain factors that occur during breast feeding must be taken into consideration. Stimulation of one breast produces immediate changes in the other breast, and in certain women the breast of one side will begin to leak as soon as extraction starts from the other; loss of fore-milk thus occurs from the second side. Furthermore, the amount taken by the baby on each side is impossible of exact regulation. Thus, in spite of all care, the two samples taken from either side cannot be exactly equivalent. Apart from these discrepancies, which were beyond our control, all other conditions were kept uniform for the two sides. A study of the figures obtained shows that in very few cases is there a general agreement in the degree of rise of fat on the two sides. Indeed it is apparent that the change of method from hand to pump for the last sample of the second side materially affects the normal rise in the second sample of the side under consideration.

Experimental results and their analysis (Tables I, II and III).

In Table I (A, B and C) are given 25 illustrative instances representing 22 women, where the effect of extracting the samples by suction with a minimum degree of pressure upon the breast was either to lessen the expected rise in fat percentage of the second sample or actually to obliterate it.

In Table I A it will be seen that there is an increase in the percentage of fat in the samples taken after the feed, whether extracted by hand or pump. This is in accordance with the findings of previous workers both in the case of cows' milk [see Eckles and Shaw, 1913; Jensen, 1906; Wellmann, 1911; Min. Agric. Fish., 1931], and human milk [see Adriance, 1897; Söldner, 1896; Schlossmann, 1900; 1902; Myers, 1927]. We ourselves have found with regard to the individual feed that in every case in which a single method of extraction was used throughout the taking of the milk, whether it were breast pump, digital pressure, or a mechanical milker[1], the later portion of the milk was richer in fat than the earlier. It is now an accepted fact that the strippings in any milk extraction show a higher percentage of fat than the middle or early milk of the same extraction.

[1] Dr Chisholm very kindly tested this method for us in Manchester.

Table I.

(The following abbreviations are used in the tables: L, left; R, right; B, before feed; A, after feed; H, hand extraction; P, pump extraction; m, month; w, week.)

Case (age of baby below)	Breast	Before feed: Volume in cc.	Before feed: Method of extraction	Before feed: % of fat	After feed: Volume in cc.	After feed: Method of extraction	After feed: % of fat	Rise in % of fat	Reduction of expected % increase (after pump extraction)
				A					
1. (H 2)	R	7	H	5·00	15	H	8·88	3·88	2·42
	L	7	H	3·79	13	P	5·25	1·46	
2. (R)	L	4·5	H	3·12	3·3	H	9·18	6·06	3·34
9 m	R	4·5	H	4·69	2·8	P	7·41	2·72	
3. (S)	R	22	H	0·73	36	H	4·19	3·46	1·54
7 m	L	15	H	3·00	14	P	4·92	1·92	
4. (T)	R	11	H	1·09	12	H	6·16	5·07	1·83
5 w	L	9·5	H	4·52	11	P	7·76	3·24	
5. (Ga)	R	18	H	3·06	23	H	5·69	2·63	1·30
3 w	L	11	H	4·22	7	P	5·55	1·33	
*6. (Co)	R	11	H	3·74	11	H	11·13	7·39	4·11
5 m 3 w	R	14	H	2·06	5	P	5·34	3·28	
				B					
7. (S. i)	R	9	H	3·97	10	H	8·73	4·76	0·64
	L	13	H	5·58	1·8	P	9·70	4·12	
8. (S. ii)	L	7	H	4·97	15	H	6·42	1·45	0·08
	R	28	H	4·13	1·5	P	5·50	1·37	
9. (J. i)	R	10	H	3·62	5·5	H	8·40	4·78	2·63
2 m	L	22	H	2·60	1	P	4·85	2·25	
10. (J. ii)	R	7·5	H	2·65	7·5	H	5·21	2·56	1·52
2 m 3 w	L	2·3	H	2·60	1·4	P	3·64	1·04	
11. (N. R.)	R	11	H	5·07	22	H	8·29	3·22	1·05
3 m	L	14	H	5·64	1·7	P	7·81	2·17	
12. (Y)	R	11	H	4·61	22	H	6·74	2·13	1·02
5 w	L	13	H	4·09	5·5	P	5·20	1·11	
13. (R. R.)	L	6·2	H	4·12	12	H	6·92	2·80	1·21
3 m	R	15	H	4·32	5·5	P	5·91	1·59	
14. (B)	R	8	H	1·26	20	H	4·93	3·67	1·20
	L	5·5	H	3·21	2·8	P	5·68	2·47	
15. (Cp)	R	6	H	2·40	20	H	5·00	2·60	1·24
3 m 3 w	L	11	H	4·08	5	P	5·44	1·36	
16. (Po)	R	9·5	H	1·28	9	H	4·60	3·32	2·47
7½ m	L	6	H	4·48	2·5	P	5·33	0·85	
17. (Sh)	L	10	H	2·29	8·5	H	7·00	4·71	3·28
	R	7	H	5·73	2	P	7·16	1·43	
				C					
18. (Ha)	R	16	H	4·73	3	H	6·60	1·87	1·85
3 m	L	6	H	6·67	3·5	P	6·69	0·02	
19. (Bl)	R	4	H	1·71	7·5	H	3·59	1·88	1·37
	L	3·5	H	2·73	3·5	P	3·24	0·51	
20. (Kn)	L	5	H	4·88	11	H	8·76	3·88	3·40
5 w	R	11	H	7·62	1	P	8·10	0·48	
21. (H. i.)	R	7	H	3·07	15	H	5·32	2·25	1·95
	L	7	H	2·88	13	P	3·18	0·30	
22. (Co)	L	5	H	3·54	6·5	H	6·30	2·76	2·72
9 w	R	4	H	2·12	2·5	P	2·16	0·04	
23. (Si)	L	7·3	H	1·66	26	H	4·70	3·04	2·59
2 m 2 w	R	6	H	6·49	10	P	6·94	0·45	
24. (G)	L	5·5	H	2·24	15	H	4·49	2·25	1·98
7 w	R	4	H	3·00	7·5	P	3·27	0·27	
25. (Ra)	R	13	H	2·01	9	H	4·91	2·90	2·60
3 m	L	12	H	5·20	3·5	P	5·50	0·30	

* As the patient had only one breast, the figures were taken on two different occasions, very close together, from the remaining breast.

Volumes. In cases 1, 2 and 4, the volumes of the samples are approximately equal. In 5 and 6 the question of the volumes falls into a grouping to be considered later. In each case the sample taken by pump contains less of the middle milk than that taken by hand.

On careful examination, however, the extent of the rise in fat will be found to be much less on the side where the final extraction was made by pump than that on the side taken by hand.

Table I B shows 11 cases where the volume obtained in the sample extracted by pump in the after-feed is very much smaller than that of the sample obtained by hand, indeed, in many cases the pump sample may be considered to consist of strippings only. In spite of this fact, it will be found that the rise to be expected in the after-sample, and shown definitely in that taken by hand, is reduced. To this table should be added cases 5 and 6 (Table I A) already referred to.

In the case of the 8 women whose samples of milk are shown in Table I C, the fat percentage of the sample taken by pump after the feed, when compared with that of the sample taken by hand before the feed, shows so small a rise (0·02 to 0·51 %) as to represent, within the limits of experimental error, practically stationary values; although on the other side at the same feed, the samples taken by hand show a consistent rise from 1·87 to 3·88 % over that taken by the same method before the feed on that side.

In certain women the process was carried even further, as will be seen in the 7 cases in Table II. Here, although on the side where hand extraction was used for both the fore- and after-samples, the normal and expected rise in the fat

Table II.

		Before feed			After feed			
Case	Breast	Volume in cc.	Method of extraction	% of fat	Volume in cc.	Method of extraction	% of fat	Rise or fall in % of fat
26. (La)	R	5·3	H	1·86	12	H	4·23	2·37
	L	6·5	H	3·16	5·5	P	3·13	−0·03
27. (Va)	R	8	H	5·83	6·5	H	9·87	4·04
	L	7	H	4·91	3·5	P	4·40	−0·51
28. (O. F.)*	L	7·5	H	1·78	36	H	5·28	3·50
	R	12	H	7·264	4	P	7·12	−0·14
29. (Fo)	L	10	H	4·81	7·5	H	9·80	4·99
	R	15	H	4·16	15	P	2·13	−2·03
30. (Sm)	L	4·5	H	1·19	6	H	4·36	3·17
	R	3	H	3·27	5·5	P	2·14	−1·13
31. (Ba)	L	4·5	H	0·64	11	H	2·33	1·69
	R	5	H	3·16	2·5	P	2·96	−0·20
32. (Wi)*	L	9·7	H	2·85	11	H	4·02	1·17
	R	9·5	H	6·26	11	P	5·89	−0·37

* In cases 28 and 32 there was a very marked difference in % of fat in "before feed" samples in the two breasts, probably due to feeding habitually from one breast only.

percentage of the after-sample occurred, the rise ranging from 1·17 to 4·99 %; on the other side where hand and pump were used for extraction, not only did no rise occur, but the percentage of fat in the after specimen was actually lower than that in the one taken before the feed.

As is usual in dealing with the human organism, the expected does not invariably occur, and we have had a few cases in which extraction by pump has not affected the percentage of fat in the sample taken after the feed in the manner to be expected. In Table III are given five such cases.

Table III.

		Before feed			After feed			
Case	Breast	Volume in cc.	Method of extraction	% of fat	Volume in cc.	Method of extraction	% of fat	Rise in % of fat
33. (Hy)	R	8	H	3·59	17	H	5·02	1·43
	L	12	H	2·62	5·5	P	4·09	1·47
34. (So)	L	7·5	H	2·69	15	H	3·62	0·93
	R	4	H	3·30	8	P	4·34	1·04
35. (Gr)	R	2·5	H	3·70	9	H	6·99	3·29
	L	10	H	3·32	6	P	6·68	3·36
36. (Ho)	R	15	H	2·50	13	H	4·17	1·67
	L	7·5	H	2·70	7·5	P	5·00	2·30
37. (Yt)	R	6	H	4·70	20	H	5·09	0·39
	L	10	H	2·42	14	P	4·28	1·86

Although the influence of the method of extraction upon the percentage of fat was shown unmistakably in all but the above cases, yet it is discernible even in these. In all except 35, it will be noted that the volumes after extraction by pump are considerably smaller than those taken by hand. In view of this fact, it would be expected that the normal rise shown by any method of extraction would be greater between a sample taken before feeding, of volume 12 cc., and a sample taken after feeding of volume 5·5 cc., than in a sample of 8 cc., before and a sample of 17 cc. after feeding. Nevertheless the actual rise in the former case is practically the same as in the latter. Indeed in cases 33, 34 and 35 the rise on both sides, within the limits of experimental error, is equal. In cases 36 and 37 we present the only cases found in our total series in which the percentage of fat in the after-sample taken by pump is significantly greater than in the sample taken by hand. It will be noted that the volumes of the after-samples in extraction by pump are considerably smaller in both cases than those taken by hand.

Deduction from above experimental data.

As has been pointed out already, the normal action of a baby feeding upon a breast can be analysed into two factors: pressure and suction; and it would appear from a study of the preceding tables that the pressure is the important factor in the production of a high percentage of fat. The greater the pressure apparently the steeper will be the rise in the fat percentage. Suction employed alone, on the other hand, tends to lower the percentage of fat. The larger, therefore, the element of suction in the act of extraction, the smaller apparently will be the ultimate rise in the fat percentage.

As will be indicated later, there appears to be an inverse ratio between the volume of milk in the breast at any time during any one feed and the percentage of fat in the sample. The larger the quantity of milk at any moment, the lower the percentage of fat in that milk—the smaller the quantity of milk, the higher the fat content. In the experiments just described, these factors have been arranged so that they act in opposition to each other.

It is suggested that the following conclusion may be formulated from these data.

In any given sample of milk, other factors being equal, the percentage of fat present will depend inversely upon the quantity of milk present in the breast at the time of taking the sample, and directly upon the degree of pressure exerted upon the areola and nipple in the process of extraction.

If this be true, then milk which has dripped from a breast, that is to say, which has been extruded in answer to conditions within the gland and not by any means of extraction from without, should theoretically give the lowest percentage of fat of all, and this is exactly what occurs.

Experimental results on dripped milk.

Table IV shows the relation of the percentage of fat in milk which has dripped from a breast to that extracted by other means. In every case it will be seen that the milk which drips is lower in its fat percentage than that obtained by extraction, whether by pump or hand.

Table IV.

b. f., before feeding; a. f., after feeding; D, dripped.

Case	Breast	Method of extraction	% of fat	Method of extraction	% of fat	Rise in % of fat
38. (Je)	R	D (b. f.)	0·26	H (b. f.)	2·94	2·68
39. (Kn)	L	D ,,	2·77	H ,,	4·88	2·11
40. (Co)	L	D ,,	1·57	H ,,	5·66	4·09
41. (Wi)	L	D ,,	1·78	H ,,	3·92	2·14
42. (Te)	R	D ,,	0·35	H ,,	1·09	0·64
43. (Pe)	R	D ,,	2·24	H ,,	5·52	3·28
44. (Wa)	R	D ,,	1·82	H ,,	2·36	0·54
45. (Ha)	R	D (a. f.)	1·12	H (a. f.)	4·02	2·90
46. (Gr)	R	D ,,	2·54	H ,,	5·75	3·21
47. (Gi)	L	D ,,	1·65	H ,,	4·49	2·84
48. (Gr)	L	D ,,	3·64	P ,,	6·16	2·52
49. (Sm)	L	D ,,	2·30	H ,,	4·36	2·06
50. (Hv)	R	D ,,	2·30	H ,,	5·02	2·72

As will be seen from Table IV, the first 7 cases show the percentage of fat in milk which has dripped to be lower than in milk taken by hand, before feeding took place or any other milk was extracted. In the last 6 cases the stimulation of suckling the baby at one breast caused the other breast to drip. These dripped samples so obtained are compared with samples previously taken from that same breast by hand or pump. It is of interest that in cases 47 and 50 milk taken by hand before feeding gave 2·24 and 3·59 % of fat, that is, 0·59 and 1·29 % more than the milk which dripped after feeding. In all other cases the fat percentage in the dripped milk was less than that in the specimen taken immediately after the feed either by hand or by pump.

It may therefore be concluded that when samples are taken from any given breast, all conditions other than the method of extraction being similar, a specimen of milk which has dripped from a breast will show the lowest proportion of fat obtainable at that moment. Nevertheless, the figure in itself need not actually be low, since the woman under examination may be one of those women who, under all circumstances, produce a high level of fat. This was shown in a case (Ra) in which the percentage of fat in the dripped milk before feeding was 4·39 rising to 6·26 and 8·63 in the before- and after-specimens taken by hand; or another case (R. R.) in which the dripped sample before feeding showed a percentage of 3·18 rising to 4·69 in the hand sample before feeding and 7·41 in the pump after-sample.

Adjustment to the method of extraction, though it occurs eventually, is of course never immediate. If a very small sample be taken by hand immediately after the dripped sample, the rise in the fat percentage, though definite, will in all probability be slight. As for instance in the case (So) when the percentage in

the sample dripped before feeding was 4·86 rising to 4·96 in the sample taken by hand before, and 6·42 in the sample taken by hand after, feeding; and another case (Pa), where the dripped sample before feeding showed a percentage of 3·15 rising to 3·55 and 5·33 in the samples taken by hand before and after feeding.

OTHER FACTORS INFLUENCING THE PERCENTAGE OF FAT IN THE MILK SAMPLE.

There are certain other factors which have an influence on the percentage of fat in the sample of milk extracted, when the same method of extraction (either digital pressure or breast-pump) is employed.

These factors have been taken into account when considering the effect of the mechanism of the extraction upon the composition of the sample.

1. *Period of lactation.* It has been found that the proportion of fat in the milk of all multiparae investigated rises between the first and fourteenth days. The nature of the rise is shown in Table V.

Table V.

Case	Day of lactation	% of fat	Case	Day of lactation	% of fat
51 (J)	2	1·40	53 (H)	6	0·44
,,	3	1·90	,,	7	3·10
,,	4	2·57	,,	8	3·35
,,	5	2·82	,,	9	3·80
,,	9	4·50	,,	10	4·60
52 (P)	5	3·26	54 (Pd)	2	2·92
,,	6	4·20	,,	5	3·40
,,	7	3·73	,,	6	3·42
,,	8	3·49	,,	7	3·88
,,	9	4·15	,,	8	4·15
,,	10	5·43	,,	12	4·58
			,,	13	4·95
			,,	14	4·72

In the case of primiparae it is found that the percentage of fat in the early part of the feed is also as a rule higher than at the end, but, as shown in Table VI, this is not invariable.

Table VI.

	Early milk of primiparæ				
			% of fat in sample		
Case	Day	Breast	Before feed	After feed	Difference
55. (F) primipara	1st	R	0·95	0·53	−0·42
,, ,,	2nd	L	1·66	1·26	−0·40
,, ,,	3rd	L	2·73	3·35	+0·62
56. (P) ,,	2nd	R and L	3·13	2·72	−0·41
,, ,,	4th	L	3·04	4·56	+1·52
,, ,,	5th	R	1·92	4·16	+2·24
,, ,,	14th	L	3·55	5·89	+2·34
57. (Pd) ,,	2nd	R	7·14	5·02	−2·12
(Small ,,	2nd	L	6·92	5·74	−1·18
quantities)	6th	R	5·44	5·60	+0·16
,, ,,	6th	L	9·98	8·66	−1·32
,, ,,	7th	R	4·36	6·10	+1·74
,, ,,	7th	L	3·64	9·02	+5·38
58. (J) multipara	3rd	R	1·57	2·12	+0·55
,, ,,	4th	L	2·57	3·09	+0·52
59. (Bk) ,,	2nd	L and R	3·50	6·05	+2·55
,, ,,	3rd	R	2·97	4·09	+1·12

2. *Individuality of women.* In the case of the milk of women examined during the first fortnight of lactation, it was found that although the mean percentage of fat in all cases rose from the first to the fourteenth day, yet in some cases the highest figure shown was between 3 and 4 %, although in other cases the percentage rose quickly to over 4 % and then to 5 % and over 6 %.

In the later periods of lactation, although two different methods of extraction were being used, the same general phenomenon was noted. In some women the lowest value for the fat obtained was rarely less than 4 % (usually between 5 and 6 %), and in some women over 7 %. In others the lowest value was less than 3 %, falling generally between 3 and 4 % and rarely rising above 4 %.

These observations suggest that some women throughout their whole lactation period will have normally a much higher percentage of fat in their milk than others. This individual variation has been observed also in the case of cows [see Min. Agric. Fish., 1931].

3. *Volume in the breast.* Emphasis has been laid in the foregoing work on the quantity of fluid obtained at any one extraction, and it might have been expected that a difference in total quantity habitually secreted would be reflected in the fat content of the milk.

Engel and Frehn [1910] consider that there is such a relationship, and that the milk output and the fat content are inversely related to one another. From a study of our own cases, there seems no such clear relationship. In Table VII are

Table VII.

Case	Age of baby	Sample	% of fat
60. (P)	First two weeks of life	2	6·17
,,	,,	3	4·22
,,	,,	4	4·50
,,	,,	7	7·40
,,	,,	8	5·82
,,	,,	9	5·20
61. (F)	,,	1	0·74
,,	,,	2	1·45
,,	,,	3	3·00
,,	,,	7	2·44

given the average percentages of fat obtained from samples from two different women on successive days, and although in each the quantity of milk was less than 10 cc. a wide difference is shown in the percentages of the fat.

If the usual quantity of fluid secreted by the breast of an individual suddenly changes, that is to say, if in a mother who habitually secretes a large amount of milk the quantity quickly decreases, or *vice versa*, then we have found that this change will affect the fat and be reflected in a rise or fall respectively. Thus, in one case when the fat percentages on the 8th and 9th days after parturition were 4·92 and 5·03 respectively, the volume of milk in the breast was small in consequence of extreme heat and profuse sweating. On the 10th day, however, after the intake of a great deal of fluid, the volume of milk in the breast suddenly rose and the percentage of fat fell to 3·8 %. The figures in Table VIII further illustrate this point.

Therefore it would appear that the final percentage of fat occurring in the milk at any given period during a feed is the product of several factors—the nature of the habitual yield of the individual woman, the place in the feed, and the mechanics of the process of extraction.

Table VIII.

Case	Volume in cc.	% of fat
62. (F)	3·3	2·73
,,	5·5	3·35
,,	20	1·52
63. (P)	Quantity under 10	6·17
,,	,,	4·22
,,	,,	4·50
,,	,,	7·40
,,	,,	5·82
,,	,,	5·20
,,	38	2·98

The above work, which was in the first instance undertaken to elucidate the variations in the fat content of milk recorded by many other workers in this field, has proved itself of value from the clinical point of view. This side of the work will be recorded in a separate communication.

Conclusions.

From our results it is suggested that the following conclusions may be drawn.

1. Differences in the method of extraction of a sample of milk appear to account for the wide variations in the fat figures hitherto obtained by different workers.

2. In any given sample of milk, other factors being equal, it would appear that the percentage of fat present will depend inversely upon the quantity of milk present in the breast at the time of taking the sample, and directly upon the degree of pressure exerted upon the areola and nipple in the process of extraction. The lowest percentage of fat will be found in milk which has dripped spontaneously from the breast.

3. There appears to be an individual variation in the average fat content of the milk of different women.

4. There would seem to be no clear relationship between a permanently high or low milk yield and the proportion of fat in the yield, though this proportion appears to be affected by sudden variations in the yield.

5. In all women investigated, the percentage of fat in the milk examined showed a rise between the first and the fourteenth days.

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CXC. THE EFFECT OF ULTRA-VIOLET, RADIUM AND X-RAY RADIATION ON GLUTATHIONE IN PURE SOLUTION.

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GLUTATHIONE has recently been shown to influence the activity of many enzyme systems [Pringsheim *et al.*, 1931; Lohmann, 1932; Waldschmidt-Leitz *et al.*, 1933] and has received considerable attention as a possible regulator of growth in the cancer cell. In view of the retarding action of radium and X-rays on cancerous growth and of the lethal effect of ultra-violet radiation of shorter wave-length than 2900 Å., the question arises as to the effect of these rays on the glutathione molecule. Accordingly the effect of such radiation on pure glutathione in aqueous or buffered solution has been investigated with regard to the fate of the sulphur in the molecule.

ULTRA-VIOLET RADIATION.

Glutathione solutions were exposed to radiation from the full quartz mercury arc[1] at a distance of 25 cm. The solutions were contained in flat dishes, giving thin layers of solution, and covered with transparent quartz plates. The dishes were surrounded by an ice-bath during the radiation to remove any possibility of destruction by heat. Samples were removed at intervals, made to 2 % sulphosalicylic acid and titrated by the iodate procedure [Woodward and Fry, 1932] for the reduced glutathione remaining. The total glutathione remaining was also determined after zinc reduction in the acid solution. By analogy with known sulphur compounds, glutathione which has been oxidised as far as its disulphoxide form (G.SO.SO.G) would be included in this figure. Table I shows the results obtained.

Table I. *Decomposition of glutathione by ultra-violet radiation.*

	Original glutathione concentration mg. per 100 cc.	Glutathione remaining after radiation: 4 hours		6 hours		8 hours	
P_H		Reduced %	Total %	Reduced %	Total %	Reduced %	Total %
3·5	50	51	75	—	—	—	—
3·8	33	50	77	—	—	—	—
7·0	33	16	63	6	—	4	40
7·3	33	0	35	—	—	—	—
6·8	200	35	62	—	—	—	—
7·5	200	14	44	12	44	—	—
8·0	200	13	52	9	48	—	—

[1] A Uviarc Laboratory Arc, 3·5–4·5 amperes, 110 volts D.C.

The solution radiated at p_H 3·5 was unbuffered, being at the p_H of pure aqueous glutathione. The other solutions were buffered in 0·02 *M* phosphate. It will be observed that there was a very rapid loss in total glutathione, that is, it was changed in such a way that it could not be reduced back to a sulphydryl compound by nascent hydrogen. Glutathione not determined by this analysis will be considered "destroyed" since it can no longer act as a member of the biologically active oxidation-reduction system, $2G.SH \rightleftharpoons G.S.S.G.$

The amount of destruction of glutathione as well as oxidation of sulphydryl is shown to be dependent upon the concentration, p_H and time of radiation. In the higher concentrations, a smaller percentage loss of glutathione was observed. The effect of p_H is shown in Table I. At a concentration of 33 mg. per 100 cc., slight changes in p_H around 7·0 produced much greater increases in both the oxidative and destructive effects than at more acid p_H values. Increasing the p_H from 7·5 to 8·0, however, in the higher concentration did not cause any greater effect. At constant p_H (7·0) oxidation of sulphydryl to disulphide glutathione proceeded much more rapidly than destruction and is, therefore, probably a primary step in the destructive effect of the radiation.

Using the closely related sulphur compounds, cysteine and cystine, Lieben and Molnar [1931] have shown a similar destructive effect of ultra-violet light.

The fate of the sulphur in the glutathione which was destroyed was investigated in the solutions of higher concentration. Free sulphate could be detected in all these by means of barium sulphate precipitation. Analytical fractionation of the forms of sulphur present was therefore made by a procedure adapted from the macro-method of Denis and Reed [1927] for sulphur compounds in blood.

Total sulphur and inorganic sulphide. No sulphur was lost as gaseous products, since 100 % was recovered as barium sulphate after oxidation by Benedict's [1909] copper nitrate oxidising reagent. Therefore no inorganic sulphide was formed. This was confirmed with lead acetate paper after acidifying and boiling the solution.

Sulphates. Both free and hydrolysable sulphate were found. There was some barium sulphate precipitation even if the solution was not boiled with acid, representing the free sulphate in the solution. This was removed after standing 20 hours, and a further precipitate of barium sulphate was obtained on gentle boiling. This was classified as the hydrolysable sulphate and is probably of the sulphonic ester type.

Intermediate sulphur. This classification includes the sulphur which was not determined in the analyses for total glutathione and total sulphate. It was

Table II. *State of sulphur after ultra-violet radiation of glutathione solutions.*

Type of sulphur compound		p_H 6·8, 4 hours %	p_H 7·5, 5 hours %	p_H 8·0, 6 hours %
Total sulphur recovered		101·0	101·5	99·5
Total glutathione	GSH			
	GSSG	62	44	48
	Sulphoxide			
Free sulphate		8·4	16·4	
Hydrolysable sulphate (sulphonate)		4·2	5·5	
Total sulphate		12·6	21·6	21·9
Inorganic sulphide		0	0	0
Intermediate sulphur	Sulphone			
	R_2S	25·4	34·4	30·1

calculated by subtracting the sum of these two determinations from 100 %, and probably includes sulphur of the organic sulphide (R_2S) and sulphone ($R—SO_2—SO_2—R$) types. No free sulphur could be detected, although all solutions were slightly yellowish after radiation.

Table II summarises the analytical results thus obtained. The destruction produced seems to be due to a progressive oxidising effect of the ultra-violet radiation rather than to liberation of inorganic sulphide or free sulphur from the molecule.

Radium.

Radon in both glass and gold seeds was used in studying its effect on glutathione. The seeds were placed directly in 10 cc. of glutathione solution, in the first case (Table III), at p_H 3·5, unbuffered and containing 50 mg. per 100 cc.; at the higher p_H values in 0·02 *M* phosphate buffer containing 33 mg. per 100 cc. The flasks were stoppered and left to stand at room temperature. Control solutions without radium were placed so as to receive no radiation from the experimental solutions (Table III).

Table III. *Effect of radium on pure glutathione.*

	Glass seeds ($\beta+\gamma$ rays)				Gold seeds (γ rays only)				
			Glutathione present after treatment			Glutathione present after treatment		Control Glutathione remaining	
p_H	Duration of treatment hours	Amount of radiation m.c.h.	Reduced %	Total %	Amount of radiation m.c.h.	Reduced %	Total %	Reduced %	Total %
3·5	25	212	75	97	—	—	—	87	100
	68	469	31	86	—	—	—	78	100
7·0	4	38	77	99	34	79	99	88	100
	24	213	0	84	191	2	92	4	100
7·3	4	32	67	95	29	70	95	65	99
	25	180	0	85	160	0	86	0	99

The results show that glutathione is extremely resistant to both the β- and γ-rays of radium. At the acid p_H, after 212 millicurie hours of treatment, there was some oxidation, but only very slight destruction. After longer treatment, 469 millicurie hours, both effects were more pronounced. At the more alkaline p_H values the autoxidation of glutathione was so rapid that the oxidative effect of the rays was not as definite. However, the destructive effect was still quite evident.

The oxidative effect observed by Hammett [1932] as expressed by a decreased nitroprusside test is thus quantitatively demonstrated.

X-rays.

Glutathione in solutions similar to those used for the radium treatment was exposed for 4 hours to unfiltered X-rays at 45 cm. distance from a machine running at 120 kv. The glutathione solution was contained in a stoppered pyrex glass flask and surrounded by ice-water for cooling during the radiation. At the more alkaline p_H values some autoxidation naturally occurs during the course of 4 hours. To allow for this a control solution was allowed to stand in ice-water for the same length of time. The results (Table IV) showed that there was no effect on the glutathione which could be attributed to X-rays. The

Table IV. *Effect of X-rays on pure glutathione.*

			Radiated solution			
			Glutathione present after radiation		Control Glutathione present	
p_H	Duration of treatment hours	Amount of radiation R units	Reduced %	Total %	Reduced %	Total %
3·5	4	15,600	95	99	100	100
3·8	4	15,600	93	104	100	100
7·0	4½	10,800	80	103	72	100
7·3	4	15,600	56	102	65	99

stability of compounds of this type toward X-rays has also been reported by Stenström and Lohmann [1928] who found no destruction of cystine in acid solution when exposed for as long as 95 hours.

Summary.

Ultra-violet radiation was found to produce a very destructive effect on glutathione, radium a slight destructive effect, both of these agents causing at the same time partial oxidation of the sulphydryl to the disulphide form. X-rays had no effect on the glutathione.

The effect of ultra-violet radiation appeared to be of the nature of an oxidative destruction. Free sulphate was formed to the extent of 42 % of the glutathione which was decomposed.

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CXCI. THE RELATION BETWEEN LIPOCHROME PIGMENTS AND VITAMIN A IN THE NUTRITION OF YOUNG FISH.

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Introduction.

The large stores of vitamin A found in the liver oils of many species of fish are of peculiar interest when it is borne in mind that frequently only traces of that substance can be detected in the food on which they normally subsist.

An explanation can be found by assuming that a fish such as the cod possesses the power to synthesise the vitamin, or, on the other hand, one can believe that the reserves merely represent an accumulation; it being remembered in the first place that although very little of the vitamin may be present in the individual organisms on which they live, most fish are voracious feeders, and, secondly, that substances such as vitamin A are readily retained by the liver.

An earlier investigation in this laboratory [Coward and Drummond, 1922] indicated that the vitamin A present in the eggs of fresh-water fish (trout) disappeared during the larval period of the development of the young fish. The evidence was, however, derived from rat-feeding tests, the technique of which at that time was much less satisfactory than it is to-day. The differentiation of vitamins A and D had not been established, and it is uncertain, therefore, in the light of recent knowledge whether the conclusions reached were justified.

It must be stated that there is growing support for the view that accumulation does occur. An investigation by MacPherson [1933] of the Dominion of Newfoundland Fishery Research Station has recently indicated that a correlation can be traced between the age of fish (length) and the reserves in the liver. Similar accumulation appears to occur also in man [Wolff, 1932; Moore, 1932].

From several points of view it appeared that the changes during the early stages of the development of young fish might be studied again with advantage, primarily because it was desired to apply the modern methods of spectroscopic assay of vitamin A, but also because we had in mind the extension of this method to the lipochrome pigments which might be biologically concerned.

The relation between carotene and vitamin A in the nutrition of mammals is now generally recognised, but there are reasons for thinking that equally important relationships in which other lipochrome pigments are concerned may be exhibited by other species.

Already it is suspected that xanthophyll, which was believed to be of far less physiological importance than carotene, may have a particular function in the growth processes of the domestic fowl and possibly also of certain herbivorous species such as the guinea-pig [Rydbom, 1933].

It appeared to be necessary, therefore, to ascertain whether any of the curious and little-studied lipochromes found so widely distributed amongst aquatic species are converted into vitamin A in the tissues of fish such as the cod. A related problem, which is not only of considerable interest but also of great practical importance, is to determine whether the red lipochromes present in such organisms as *Calanus* or *Euphausia*, which incidentally are known to differ in several respects from the recognised pigments of that class, are the origin of the large amount of vitamin A found in the livers of those species of whales which feed almost exclusively on zooplankton.

Experimental.

A convenient material for this investigation was available in the form of the fertilised eggs of trout. Both brown trout and rainbow trout were employed. The technique of hatching the eggs and of raising and feeding the young fish was that described in the communication already referred to [Coward and Drummond, 1922]. Periodically during the larval period a number of the young fish were removed, killed and the fatty materials extracted and examined. As soon as the fry were able to take solid food they were divided into three groups and fed on the following diets:

Group I. Pulped rat-liver.
Group II. Finely divided fresh cod-muscle.
Group III. Cod-muscle and fresh-water green algae.

The object of the third group was to ascertain whether the very young fish could convert the carotene or xanthophyll of the green algae into vitamin A, there being evidence that normally at this early period of their development they ingest a certain amount of "green" food. The alga employed was a motile form of *Chlamydomonas*, cultivated in tap-water[1]. A culture of it was poured into the circulating water in the trout "ponds" each day at the same time as the finely divided cod-muscle was being given. Microscopic examination of fish revealed that green cells had been swallowed and digested but we had no means of judging how much was eaten. We suspect that it was quite a small amount by comparison with the other food consumed.

Again, at intervals specimens were taken for extraction and examination of the fats. In Fig. 1 there is plotted the absorption spectrum of the extract pre-

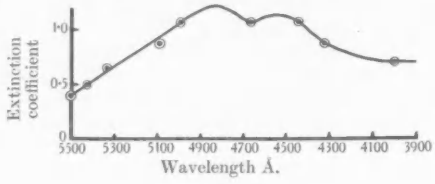

Fig. 1.

pared from the eggs of the brown trout. The absorption in the visible region does not entirely correspond with that of carotene. Saponification of the extract and application of the phase test revealed that the orange-red pigment is preferentially soluble in the light petroleum layer and, therefore, presumably it is a hydrocarbon or, at any rate, a lipochrome of low polarity.

[1] We are indebted to Prof. Salisbury for identifying the organism we employed.

A comparison of this curve with that recorded by Lovern and Morton [1931] for a pigment found in monk fish-liver oils shows a striking similarity.

The exhaustive researches of Lönnberg and Hellström [1932] on the pigments of fish suggest that this fat-soluble colouring matter may be widely distributed.

The absorption spectrum of the extract from the eggs of the rainbow trout is similar to that of the brown trout eggs, but much less pigment is present.

In Table I the absorption at 4800 Å. near the maximum of the carotenoid band and the absorption at 3280 Å. have been evaluated for the brown trout. The figures express the extinction coefficient for a concentration where 1 cc. of extract ($CHCl_3$) is equivalent to 1 g. of fish.

Table I.

1	2	3	4	5	6	7	8	9
Group	Days from commencement	Absorption at 4800 Å.	Absorption at 3280 Å.	Corrected value at 3280 Å.	Weight	Weight × absorption at 4800 Å.	Weight × corrected absorption at 3280 Å.	Column 7 + column 8
Eggs	0	2·6	2·0	0	0·083	0·215	0	0·215
Larval trout with egg sacs	6	2·2	2·08	0·39	0·091	0·200	0·0355	0·236
	15	2·2	2·10	0·41	0·096	0·211	0·0394	0·250
	23	1·8	1·80	0·41	0·108	0·204	0·0442	0·248
	30	1·1	1·4	0·55	0·136	0·150	0·0748	0·225
Feeding begun:								
Group I	37	0·98	1·96	1·21	0·126	0·124	0·1525	0·277
	51	1·12	2·08	1·22	0·104	0·117	0·1269	0·244
	62	0·70	2·10	1·57	0·140	0·098	0·2198	0·318
	76	0·325	5·20	4·95	0·165	0·054	0·8168	0·871
	92	0·82	10·70	10·07	0·162	0·133	1·6313	1·734
– A for 16 days	92	0·72	8·4	7·85	0·143	0·103	1·1221	1·225
Group II	37	0·82	1·3	0·67	0·152	0·125	0·1018	0·227
	51	1·06	1·9	1·08	0·104	0·110	0·1123	0·222
	62	0·45	1·92	1·57	0·120	0·054	0·1884	0·242
	76	0·35	1·62	1·35	0·138	0·048	0·1863	0·234
Group III	37	0·98	1·20	0·55	0·128	0·125	0·0704	0·195
	51	1·06	1·85	1·03	0·104	0·110	0·1071	0·217
	62	1·15	2·22	1·33	0·095	0·109	0·1264	0·235
	76	0·40	2·01	1·70	0·120	0·048	0·204	0·252
						Mean for eggs and Groups II and III:		0·231

While the absorption at 4800 Å. is a measure of the concentration of pigment, the figure obtained at 3280 Å. must be regarded as probably compounded of three components—the absorption of any vitamin A present, the absorption due to the pigment and that due to other irrelevant chloroform-soluble materials. If the absorption of the pigment in this region is subtracted from the gross absorption, the resulting values represent changes in the content of vitamin A provided that the irrelevant substances are present in constant concentration. This latter requirement has been assumed in the results to be described, but the possibility of variations in concentration of substances other than vitamin A absorbing at 3280 Å. cannot be disregarded.

It was necessary, therefore, to allow for the absorption at 3280 Å. of the pigment, but since there was always the possibility of the presence of some vitamin A in the extracts of the eggs, an upper limit only could be set for this absorption, *i.e.* the extinction coefficient where the ratio of absorption at

3280 Å. to absorption at 4800 Å. is at a minimum. This condition was fulfilled in the earliest experiment on the eggs.

It will be seen (Table I) that the ratio is 0·77, so that we have assumed that at this stage the vitamin A absorption is zero, and all subsequent values at 3280 Å. have been "corrected" in a similar manner by subtracting from them 0·77 of the absorption at 4800 Å. It cannot be claimed that the "corrected" values express true absorption due to vitamin A alone, but we have eliminated by this means any variations brought about by the pigment, so that those which do occur probably arise from varying concentration of vitamin A. By multiplying this value by the mean weight of the fish, figures are obtained which represent the amounts of carotenoid (column 7) and of vitamin A (column 8) present in each fish.

In Table I it will be seen that in the young fish before feeding began the content of pigment decreased, but that this was accompanied by a corresponding rise in vitamin A content. This state of affairs continued throughout the tests on groups II and III, the two types of food showing little difference. In column 9 the sums of the values contained in columns 7 and 8 are given and these show remarkable constancy, indicating that as absorption at 3280 Å. increases, an approximately equal decrease at 4800 Å. occurs. One explanation of this would be that the pigment is being converted into vitamin A. Now $E^{1\%}_{1\,\mathrm{cm.}}$ for carotene is about 2500 at 4800 Å. [McNicholas, 1931], while for the purest vitamin A preparation $E^{1\%}_{1\,\mathrm{cm.}}$ is 1600 [Carr and Jewell, 1933]; thus we should expect for efficient conversion of carotene into vitamin A a rather greater decrease in absorption at 4800 Å. than the increase at 3280 Å. Since it is reasonable to expect that the pigment of the trout is roughly of the same absorbing power as carotene, the conversion appears to be an efficient one.

Group I fed with rat-liver showed a substantial increase both in weight and in vitamin A content as compared with the fish fed on cod-muscle. When the former were deprived of liver and were given only cod-muscle for 16 days they decreased considerably in weight and showed a reduced amount of substance absorbing at 3280 Å.

The results with the rainbow trout are expressed in the form of graphs. In Fig. 2 the superior growth on the liver diet is well marked and there is

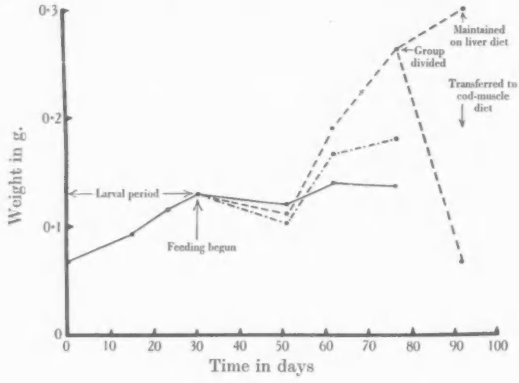

Fig. 2.

•—• Cod-muscle diet. •— —• Rat-liver diet. •— · —• Cod-muscle diet + algae.

evidence that supplementing the "white fish" diet with green algae was beneficial. From the curves in Fig. 3, there is also an indication of storage of carotene

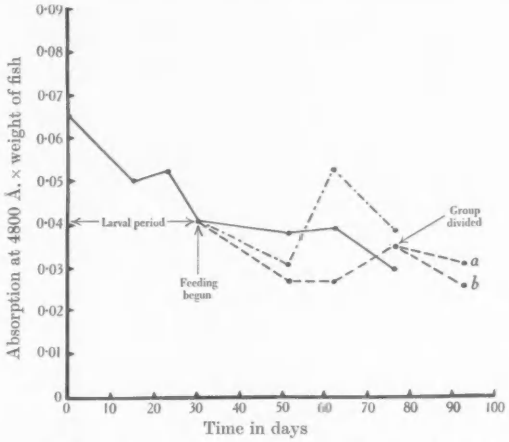

Fig. 3. (*a*) Maintained on liver diet, (*b*) transferred to cod-muscle diet.
•—• Cod-muscle diet. •— · —• Cod-muscle diet + algae. •— —• Rat-liver diet.

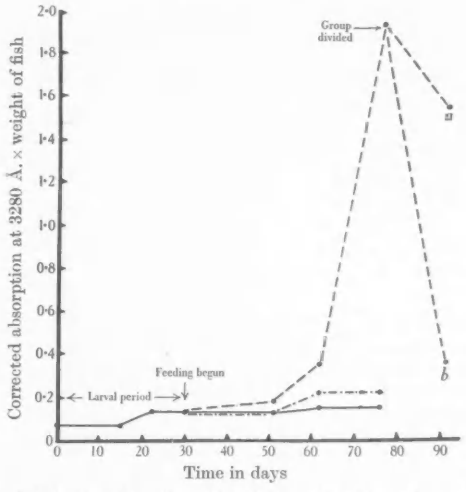

Fig. 4. (*a*) Maintained on liver diet, (*b*) transferred to cod-muscle diet.
•—• Cod-muscle diet. •— · —• Cod-muscle diet + algae. •— —• Rat-liver diet.

or a similar pigment by the fish in group III. In Fig. 4 the vitamin A content in the group fed with rat-liver is shown to rise very steeply, and to fall as sharply when deprived of vitamin A for 16 days by feeding exclusively on the cod-flesh

diet. The concentration attained would appear therefore to be abnormally high. Corresponding with the rapid decline in the amount of vitamin A in the case of fish deprived of liver is a sharp decrease in weight (Fig. 2).

It will be noticed that in groups II and III the reciprocal effects of decreasing pigmentation and increasing nett absorption at 3280 Å. indicated in Table I, are also apparent (Figs. 3 and 4). It would seem, however, that the rainbow trout eggs originally possess less pigment but more vitamin A. The mean value for the constant corresponding to that in column 8 of Table I is considerably lower (0·181). For group I this figure rapidly increased until it was greater than for the brown trout. The relative vitalities of the two kinds varied in like manner. In the initial stages the rainbow trout had a high mortality but in later stages they were more hardy than the brown variety, a fact which would indicate that the "constant" referred to may be a measure of vitamin A reserves.

It is apparent that our conclusions are at variance with those derived from the previous investigation in this laboratory [Coward and Drummond, 1922] but as we have already remarked, the feeding tests which formed the basis of those experiments are open to criticism in the light of present day knowledge.

Summary.

1. The ova of brown and rainbow trout contain a pigment similar to, but not identical with, carotene. It is probably a hydrocarbon[1].

2. In the development of the young fish the amount of this pigment decreases, and there is a corresponding increase of absorption at 3280 Å., suggesting a conversion into vitamin A.

3. Indications were obtained that the lipochrome content of young fish may be increased when they are fed on a diet containing a typical green alga (*Chlamydomonas*). It is uncertain whether the pigment stored is unchanged carotene or a substance showing similar absorption.

[1] [*Note added in proof.*] Kuhn and Lederer [*Ber. deutsch. chem. Ges.* 1933, **66**, 488] have recently described a pigment "astacin" isolated from the lobster. Its formula is given as $C_{27}H_{32}O_3$ and a lactone structure is indicated by the fact that its distribution in the phase test can be reversed by changing the reaction. It is possible that this pigment is related to that we have described.

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CXCII. THE MICRO-DETERMINATION OF PENTOSES, FREE AND COMBINED. I.

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Recent investigations have shown the widespread distribution of pentoses in combined form within the animal organism. In many cases the amount of material available for analysis is small and as it is frequently necessary to carry out rapidly a large number of analyses, it is advisable to have at one's disposal a simple and reliable micro-method. The majority of modern methods depend upon the conversion of pentoses into furfuraldehyde on distillation with 12–20 % hydrochloric acid [Gans *et al.*, 1888] and the determination of the amount of furfuraldehyde, either gravimetrically after condensation with phloroglucinol in acid solution or colorimetrically with aniline and acetic acid. The colorimetric method has the great advantage of detecting furfuraldehyde in extreme dilutions. Youngburg and Pucher [1924] examined carefully the conditions under which the reaction between furfuraldehyde and aniline acetate requires to be carried out in order to obtain accurate quantitative results. Pervier and Gortner [1923] had already directed attention to the advantages of steam-distillation in the removal of furfuraldehyde from the reaction mixture, and Hoffman [1927] employed this method with certain modifications, along with the colorimetric technique of Youngburg and Pucher. Steudel and Wohinz [1931] and many others have adopted, with slight modifications, the Hoffman method. Youngburg [1927] later replaced hydrochloric with phosphoric acid, removing the furfuraldehyde in the usual way by steam-distillation, and obtained the theoretical yield from *d*-xylose, 78·5 % of the theoretical from *d*-ribose, 64·9 % from *l*-arabinose and *d*-arabinose and 52·2 % from *d*-lyxose. He obtained very small yields (1–6 %) from various hexoses, disaccharides and polysaccharides, glucose furnishing 4·84 %.

The recent literature shows that even when all precautions are taken as regards removal of furfuraldehyde from the reaction mixture the yields obtained by different investigators from the same pentoses, free or combined, are far from uniform. All are agreed that the yield from the pyrimidine nucleotides is extremely small. The most recent results of furfuraldehyde yields from various purine nucleotides and nucleosides of yeast and of animal origin are those given by Steudel [1933].

The great difference in the furfuraldehyde yields between the yeast and the animal nucleotides is very striking and has naturally attracted a great deal of attention. The rise in the furfuraldehyde yield after deamination and removal of the phosphoric acid from the muscle adenylic acid has naturally been interpreted as due to a difference in the structure of the ribosephosphoric acid in the yeast and the muscle nucleotides. Embden and Schmidt [1929] were the first to show that there are striking differences between the muscle adenylic acid and the yeast

adenylic acid, among which may be mentioned solubility, optical rotation, rates of acid hydrolysis of the phosphoryl linkage, deamination (chemical and enzymic) as well as furfuraldehyde yields. Recently Klimek and Parnas [1932] have drawn attention to a difference as regards the formation of a complex copper salt with muscle adenylic acid and not with the yeast derivative.

That such differences are due in part to the position of the hydroxyl groups and the phosphoryl linkage of the pentose is extremely probable, but it is difficult to ascribe the very great disparity in the furfuraldehyde yields to such a cause.

The principle of the micro-method which we have employed during the last two years is briefly the production of furfuraldehyde from the action of acetic acid on pentoses, free and combined, in evacuated and sealed tubes kept at 170° for some hours, and the subsequent direct determination of the furfuraldehyde colorimetrically with aniline acetate. Numerous experiments were carried out in order to determine the conditions which led to maximum yields of furfuraldehyde.

(1) *Acids.* Many acids in different concentrations were tested, *e.g.* oxalic, phosphoric, acetic, acetic *plus* trichloroacetic and others. Finally it was found that acetic acid alone gave the best yields and that the most favourable concentration was approximately 50 %. This may not be the exact optimum, but such a concentration is more convenient to work with than one slightly stronger or weaker.

(2) *Temperature.* The optimum temperature was found to be about 170° in order that the maximum yield should be obtained within 2–4 hours.

(3) *Evacuation of the tubes.* Good evacuation (water- or oil-pump) is absolutely essential in order to avoid destruction of furfuraldehyde. Furfuraldehyde solutions heated under the standard conditions show no sign of any destruction, the recovery being complete.

(4) *Concentration of solutions.* Different concentrations covering a wide range were tested. Dilute solutions (1·0 cc. = 0·05 mg. furfuraldehyde) were found on the whole to be most suitable.

The technique of the method.

The aqueous solutions of the pentoses and of the purine nucleotides and their derivatives are usually made of such a concentration that 1·0 cc. of each solution should give a theoretical yield of 0·1 mg. furfuraldehyde. 50 cc. of the solution are then run into a 100 cc. flask and made up to 100 cc. with glacial acetic acid. The theoretical yield of furfuraldehyde from this 50 % acid solution is therefore 0·05 mg. per cc. Three to 4 cc. of the acid solution are then transferred with a capillary pipette into glass tubes 1 cm. in diameter and 16–18 cm. in length. The closed ends of the tubes are previously blown into small bulbs to facilitate evacuation. The tubes are then drawn out, evacuated and sealed. They are now covered with asbestos paper, placed in iron or lead tubes and heated in an electric oven at the temperatures and for the times found to be most suitable. The tubes are finally allowed to cool and opened with the usual precautions. The colorimetric determination is then made in the usual way, using as a standard the one employed by Youngburg and Pucher, the solutions for comparison being made up as follows:

I. The reaction mixture.	II. Series of fractions of standard furfuraldehyde solution (1·0 cc.–0·05 mg. furfuraldehyde).
1·00 cc. of the solution.	(1) 0·70 cc., (2) 0·80 cc., or (3) 0·90 cc. of standard furfuraldehyde.
0·5 cc. aniline.	0·5 cc. aniline.
3·5 cc. glacial acetic acid.	4·0 cc. glacial acetic acid.
Distilled water up to 10 cc.	Distilled water up to 10 cc.

After the reaction mixture and series of standards have stood for 15 minutes in the dark, they are compared, and the standard (1), (2) or (3), which most closely approximates to the colour of

the unknown is matched with it in the colorimeter. When dealing with reaction mixtures of widely varying furfuraldehyde concentrations, the final procedure of Youngburg and Pucher [1924, p. 745] was adopted in its entirety. All measurements and reaction mixtures were made with standard 1·0 cc. pipettes divided into 0·01 cc.

The stock furfuraldehyde solution is made up in water saturated with toluene so that the strength is 5·0 mg. furfuraldehyde per cc. and is stored in the ice-chest. From this the dilute standard can be prepared as required.

A few examples from a very large number are given in Table I in order to show why a concentration of 50 % acetic acid at 170° for a period of 3–4 hours has been chosen as most satisfactory.

Table I. *Yields of furfuraldehyde from pentoses, nucleotides and nucleosides under various conditions.*

Substance	Conc. of acetic acid %	Temp. ° C.	% yield of furfuraldehyde after 1	1·25	1·5	1·75	2	3	4	5	6 hrs.
d-Xylose (*a*)*	50	170	66·5	—	—	—	84·5	86·7	92·9	93·1	80·6
,, (*b*)	50	170	—	—	—	—	86·0	88·8	91·8	—	—
,, (*b*)	50	180	76·4	85·1	88·9	82·2	80·5	—	—	—	—
,, (*a*)	25	170	—	—	—	—	—	72·1	77·1	63·1	—
,, (*a*)	75	170	50·3	—	—	—	64·5	—	—	52·7	—
l-Arabinose (*a*)	50	170	—	—	—	—	62·3	73·4	81·3	68·6	—
,, (*b*)	50	170	—	—	—	—	69·8	70·2	70·2	—	—
,, (*b*)	50	180	—	—	76·4	—	63·8	—	—	—	—
Yeast adenylic acid (*a*)	50	170	65·9	—	—	—	84·0	85·5	95·1	83·5	76·9
,, (*b*)	50	170	—	—	72·6	—	81·1	82·7	81·6	71·4	—
,, (*b*)	50	180	67·0	84·5	74·4	—	—	—	—	—	—
Yeast adenosine (*a*)	50	170	74·5	—	—	—	—	89·2	—	—	82·5
,, (*b*)	50	170	—	—	—	—	75·0	85·6	82·1	72·6	—
,, (*b*)	50	180	72·4	76·3	71·4	—	—	—	—	—	—
Yeast guanosine (*a*)	50	170	—	—	—	—	64·4	—	—	—	—
,, (*b*)	50	170	—	—	—	—	70·9	75·5	—	68·2	—
,, (*b*)	50	180	—	72·4	—	—	68·2	—	—	—	—
Yeast guanylic acid (*b*)	50	170	—	—	—	—	67·7	78·9	72·6	—	—
,, (*b*)	50	180	72·6	72·9	71·4	—	—	—	—	—	—
Muscle adenylic acid I (*b*)	50	170	60·2	—	—	—	73·0	62·4	59·8	—	—
,, II (*b*)	50	170	—	—	—	—	67·5	75·1	61·6	—	—
Ba inosinate (*b*)	50	170	—	—	—	—	76·0	—	—	—	—
Ca adenosinetriphosphate (heart muscle)	50	170	62·5	—	—	—	55·5	55·5	62·5	—	—
Ditto (rabbit's skeletal muscle)	50	170	53·8	—	—	—	53·1	49·1	—	—	—
Ditto (frog's skeletal muscle)	50	170	44·6	—	—	—	—	—	—	—	—

* (*a*) and (*b*) signify concentrations of substance yielding theoretically 0·1 and 0·05 mg. furfuraldehyde per cc. of solution.

Note. We are indebted to Dr Steudel for the adenylic acid (I), to Dr Henning for the same substance (II), to Dr Levene for the Ba inosinate and to Dr Strain for the specimen of adenosinetriphosphate from the heart muscle of the horse; to all of whom we offer our best thanks.

The furfuraldehyde yields from the muscle nucleotides are very much higher than those which have been obtained by others using the distillation method [*cf.* Steudel, 1933], so that differences between the nucleotides of yeast and of muscle origin are by no means so evident as one had been led to expect. Further work on this subject is being carried out.

Furfuraldehyde yields from the pyrimidine nucleotides.

A specimen of cytidylic acid supplied by the British Drug Houses Ltd., gave the following yields on heating with 50 % acetic acid at 170°.

3 hours, 27·5 %; 5 hours, 29·4 %; 7 hours, 28·2 %.

Furfuraldehyde yields from hexoses and sucrose.

In dilute solutions there was practically no furfuraldehyde production, the colour development being so slight as to make the readings impossible. In much more concentrated solutions than those used for pentoses, glucose and sucrose gave apparently small yields, 4·18 and 4·56 % respectively. It is probable however that the colour development is due to the presence of hydroxymethylfurfuraldehyde. Slight humin formation also masks the colour change and renders colorimetric comparison unsatisfactory.

It is evident that there are variations in the maximum yields of furfuraldehyde obtained by this method, depending as always on the difficulty of obtaining maximum production of furfuraldehyde with minimum destruction, but in our opinion the same is true of all acid distillation methods. This is shown by a comparison of the yields obtained by Hoffman and Steudel respectively, using approximately the same procedure. The method here described can be readily carried out and one may easily compare and determine with accuracy the yields under exactly the same conditions from a pure pentose of known concentration and pentose in some form of combination. With the qualifications which follow from the above remarks, one may give provisionally as approximate maximum yields of furfuraldehyde from pentoses, *etc.* which have been examined the following values.

	%
d-Xylose	90–93
l-Arabinose	75–81
Yeast adenylic acid	85–95
Yeast adenosine	80–90
Yeast guanosine	70–75
Yeast guanylic acid	70–80
Muscle adenylic acid	70–75
Muscle inosinic acid	75
Muscle (skeletal) adenosinetriphosphoric acid	50–53
Muscle (cardiac) adenosinetriphosphoric acid	55–63
Cytidylic acid	27–29
Hexoses (in dilute solution)	0

Summary.

A micro-method for the determination of pentoses, free and combined, is described, and the conditions under which maximum yields may be obtained are discussed.

By this method the furfuraldehyde yields from the purine nucleotides of muscle are found to be much higher than those obtained by the usual steam-distillation method from a hydrochloric acid reaction mixture.

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CXCIII. THE INFLUENCE OF COD-LIVER OIL ON THE HAEMOLYTIC COMPLEMENT OF HUMAN BEINGS.

By THOMAS WILLIAM BRYANT OSBORN.

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In a previous communication [Osborn, 1931], it was shown that the complement of rats fed on a diet deficient in vitamin A tended to be lower than that of control animals receiving cod-liver oil. The aim of the present work was to discover whether a dose of cod-liver oil produced a change in complement concentration in human beings. Fifty cases were experimented on, and they were compared with fifty cases receiving olive oil. A large dose of 50 cc. was given in each case in order to obtain the maximum possible effect. This was taken without difficulty by all the subjects except one. A Zulu who had a superstitious objection to fish of any sort and who detected the fishy flavour of the cod-liver oil vomited a few cc. of his dose.

In all 100 experiments were made:

14 on Oxford students and research workers,
24 on South African students,
42 on natives in the Johannesburg Hospital,
20 on natives in the City Deep Mine Hospital.

Fourteen individuals submitted to both the cod-liver oil and the olive oil, with an interval of a day or more between the experiments. In the other experiments alternate individuals were given cod-liver oil and olive oil respectively. The students were all normal healthy individuals. The hospital cases were convalescents, usually a day or two before their discharge, fractures and other simple surgical cases uncontaminated by sepsis, and others similarly free from any acute infection.

In each experiment about 0·3 cc. of blood was withdrawn from the finger tip into a small agglutination-tube between 8 and 10 a.m. The dose of oil was then given and one or more further samples of blood were taken from one to five hours after the dose.

The tubes were stoppered and allowed to stand at room temperature for 24 hours. The complement activity of the resulting serum was then estimated by measuring the time taken for complete haemolysis of a fixed volume of sensitised sheep's corpuscles by a measured volume of the serum. Two observations were made on each sample of blood. The S.A. Institute for Medical Research kindly arranged for a regular supply of the corpuscles. They were sensitised with five units of anti-sheep corpuscle serum and were suspended in normal saline to give a 3 % suspension. The details of their preparation are as follows.

2 parts of sheep's blood are drawn into 1 part of a 2 % solution of sodium citrate. The corpuscles are deposited by centrifuging and are washed three times

with 3 volumes of 0·85 % NaCl, being centrifuged for 10 minutes at 3000 r.p.m. between each washing. A volume of corpuscles, measured by means of a pipette, is then suspended in sufficient 0·85 % saline to give a 3 % suspension. To this are added 5 units of anti-sheep corpuscle serum (*i.e.* five times the minimum volume needed for complete haemolysis in the presence of complement). The mixture is then allowed to stand for at least an hour.

A capillary pipette described in the Appendix was used for the measurements. The following volumes were drawn up in the order stated with a bubble of air between each:

	1·0 vol.	normal saline
	1·0 ,,	normal saline
	x ,,	serum diluted three or four times with normal saline
	$1-x$,,	normal saline
	0·5 ,,	normal saline
	0·5 ,,	3 % suspension sensitised sheep's corpuscles
Total	4·0 vol.	

In the pipette used 1 volume was equal to about 50 mm.^{3} Since all the measurements were made with the same pipette, the absolute volume is of no importance. The measured volumes were expelled in the opposite order into an agglutination-tube. This procedure ensures that the serum is washed out of the pipette by two volumes of saline, and that it is separated from the sensitised corpuscles by two small volumes of saline. The solutions were mixed thoroughly, and the tube was warmed in a glass-sided water-bath at 37°. A white glazed tile, ruled with black lines, was placed behind the tubes in the bath, and they were illuminated with a strong beam of light. In this way it was possible to estimate, to within a few seconds, the point of complete haemolysis. Since a reaction taking longer than about 4 minutes has a less definite end-point, the dilution of the serum and the volume of the dilute serum used (x in the list of reagents), were adjusted to give a haemolysis time of 2–3 minutes.

The activity of the complement after the dose was expressed as a percentage of the strength before the dose.

$$\frac{\text{Activity of complement after dose}}{\text{Activity of complement before dose}} \times \frac{100}{1}$$

$$= \frac{\text{Speed of haemolysis after dose}}{\text{Speed of haemolysis before dose}} \times \frac{100}{1}$$

$$= \frac{\text{Time for complete haemolysis before dose}}{\text{Time for complete haemolysis after dose}} \times \frac{100}{1}.$$

For these times to be comparable it was obviously necessary for the same volume of serum to be used in the estimation of the activity before and after the dose, *i.e.* in any one comparison the dilution of the serum and the volume of the diluted serum (x) were the same.

In a few of the cases samples of blood were taken hourly from 1 to 5 hours after the dose, in others a single sample was taken at from $2\frac{1}{2}$ to 4 hours. In all 174 estimations were made following cod-liver oil and 170 following olive oil. The results are expressed in the form of a histogram in the accompanying diagram. 100 %, marked by the heavy black line, is the strength of the complement before the dose.

Protocol of one pair of observations:

Subject: J. H. G.

Date: 18th Jan. 1933.

Blood sample taken at 9.30 a.m., dose of 50 cc. of cod-liver oil given immediately after, second sample taken at 1 p.m., food and violent exercise being avoided in the interval. Blood stood at room temperature overnight to allow separation of the serum. Serum diluted with 3 volumes of 0·9 % saline.

	9.30 a.m. blood	1 p.m. blood
Volume diluted serum	0·6	0·6
Volume sensitised corps.	0·5	0·5
Volume 0·9 % saline	2·9	2·9
Haemolysis time	168 secs.	145 secs.
Percentage of original speed	—	$\frac{168 \times 100}{145} = 116\ \%$

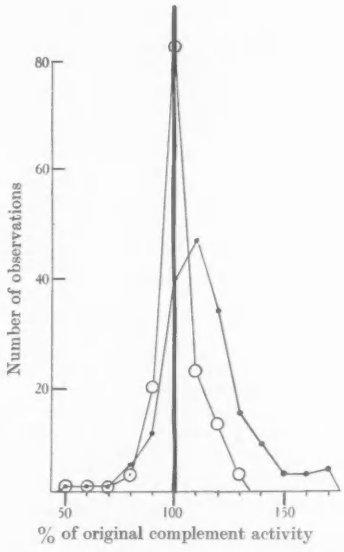

Fig. 1. Histogram showing complement activity of serum of individuals after taking cod-liver oil and olive oil. Expressed as percentage of activity of serum taken before dose.

The statistical analysis of the figures gives the following results:

Mean of cod-liver oil	= 113·4
Mean of olive oil	= 102·7
Difference	= 10·7
Standard deviation of cod-liver oil ($\sigma_{C.L.O.}$)	= 19·0
Standard deviation of olive oil ($\sigma_{O.O.}$)	= 10·8

Standard error of difference ϵ_{12}

$$= \sqrt{\frac{\sigma_{C.L.O.}^2}{N_{C.L.O.}} + \frac{\sigma_{O.O.}^2}{N_{O.O.}}} = 1{\cdot}66.$$

Since the deviation of the means is approximately 6·5 times the standard error of the difference, it seems to be significant.

Discussion.

It is an accepted fact that haemolytic complement is not a single substance. The work of Buchner [1893], Ferrata [1907], Sachs and Teruuchi [1907], Brand [1907], and others showed that it contained two thermolabile parts, the "Midstuck" and "Endstuck." The work of v. Dungern [1900], Ehrlich and Sachs [1902], Coca [1914], Hyde [1925] and others led to the recognition of a third part, while Gordon, Whitehead and Wormall [1926] produced good evidence of a fourth part. In these circumstances it would not be expected that an increase in the concentration of one of the parts would invariably lead to an increase in the total complement activity. This would only occur if the part increased were the limiting factor in the blood in question. This supposition may explain the unevenness of the results obtained. While the administration of cod-liver oil caused an increase as compared with the olive oil when measured by the average effect, and while this increase was well marked in a percentage of cases, it did not occur invariably. This is what might be expected by our previous reasoning. If some factor, present in cod-liver oil and absent from olive oil, caused an increase in one of the parts of the complement complex, an ingestion of complement would cause an increase in the total complement activity in some cases but not in others.

These results are interesting when taken in conjunction with the previous results [Osborn, 1931], which showed that rats fed on a diet deficient in vitamin A had, on the average, a lower complement than control rats receiving cod-liver oil.

Summary.

The ingestion of a dose of cod-liver oil by 50 experimental subjects produced, on the average, a rise in haemolytic complement which was greater than that produced by olive oil. The significance of the fact that this rise took place in some cases but not in others is discussed.

The author's thanks are due to Prof. J. P. Dalton, who kindly undertook the statistical analysis of the results, to Dr G. Buchanan of the S.A. Institute for Medical Research for the supply of blood corpuscles, and to the subjects who allowed a rather unpleasant experiment to be performed on them.

Appendix.

The instrument described below has been found to be suitable for the repeated measurement of volumes of liquid of the order of 5 mm.^{3}

A piece of tubing is drawn out into the shape shown in Fig. 2. A small drop of mercury, equal in volume to the smallest graduation required, is introduced at the broad end *D* and allowed to run round into the capillary *A*. A rubber teat is filled with mercury and pushed over the broad end *D*. Pressure on the teat is regulated by means of a screw-clip and in this way the column of mercury in the capillary *A* may be moved down the tube and the required number of graduations marked on the tube in Indian ink or glass paint. The instrument is then clamped in a vertical position. A second clamp holds the rubber teat. Liquids are drawn into the pipette by relaxing the pressure on the teat and expelled from it by increasing the pressure.

For accurate measurement the elasticity of the air within the instrument is a disadvantage and therefore the volume of this air is reduced as much as possible. It is for this reason that *C* is drawn out into a capillary and that the teat is filled with mercury.

In making mixtures of different volumes a method is used which is described by Wright. The measured volumes are separated by columns of air in the capillary. If, for instance, it is required

to mix 1·7 volumes of *A* with 0·6 volumes of *B* by means of a pipette holding 1 volume and graduated into 10 divisions, the following are drawn up into the pipette:

1·0 volumes *A*,
a small column of air,
0·7 volumes *A*,
a small column of air,
0·6 volumes of *B*.

These are then all expelled together. They may be mixed by being repeatedly drawn in and out of the pipette. Another convenient method is to blow a small bubble of air into the mixture and rotate it about.

The small bulb *B* prevents liquids being drawn over into the mercury in *D*.

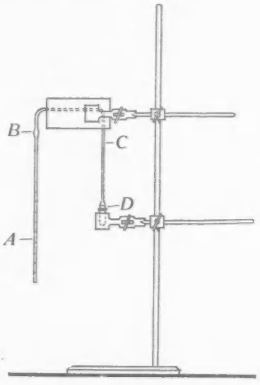

Fig. 2. Diagram of pipette.

Wright [1921] advocates the coating of the inside of small pipettes with wax to obviate the error produced by wetting the glass. The author has not, up to the present, found a wax which will hold to the walls for any length of time. If such a substance could be found, it would be a great advantage in all volumetric micro-chemical work. An alternative suggestion of Wright's has, however, been found to be helpful. If the external surface of the tip of the pipette has a coating of wax the removal of the last drop of liquid is facilitated.

In most cases one pipette would be used for all measurements, therefore the absolute volume of the graduated part of the pipette need seldom have any definite value. Provided it is divided into a number of equal parts, relative volumes can be measured accurately.

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CXCIV. HIGH PROTEIN DIETS AND ACID-BASE MECHANISM.

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ALTHOUGH it has been widely held, particularly since the experiments of Chittenden and of Chalmers Watson, that the presence in the food of high proportions of protein is harmful, there is still much conflict of evidence as to whether this is really so in the case of omnivorous species. Many investigators have attempted to analyse the underlying effects of increased protein rations, particularly with regard to the effect of protein in producing kidney disorders. The primary reason for undertaking the following experimental work was to determine whether the adverse effect which has been reported by some investigators was due to the acidogenic properties possessed by protein in virtue of its content of sulphur and phosphorus[2]. This acidogenic effect has been demonstrated in man by Farquharson *et al.* [1931] who found increased excretion of ammonia and loss of calcium in response to a high protein diet. As to other effects of high protein diets in man, though such diets tend to alter the proportion of putrefactive organisms in the intestine [Sanborn, 1931; Torrer and Montu, 1931], the work of Krogh and Krogh [1913] and Thomas [1927] on eskimos, and that of McClellan and DuBois [1930], McClellan *et al.* [1930], and Tolstoi [1929] on trained subjects, all fail to indicate serious harmful effects from excessive dietary protein, whilst physical inferiority results in the case of African and of Bengali tribes if the protein is low [Orr and Gilks, 1931; McCay, 1912].

In animals the presence of excess of protein in the food has been found to cause renal hypertrophy by many workers [Osborne *et al.*, 1926–27; Hassan and Drummond, 1927; Reader and Drummond, 1925; 1926; Drummond *et al.*, 1922; Jackson, 1924–25; Jackson and Riggs, 1926; Francis *et al.*, 1931; Stewart, 1929; Longwell *et al.*, 1932; MacKay *et al.*, 1928]. Actual renal damage has been observed by Newburgh *et al.* [1919; 1923; 1928; 1931], Polvogt *et al.* [1923], Blatherwick *et al.* [1931], Maclean *et al.* [1926], Evans and Risley [1925], Smith *et al.* [1927], Jackson and Moore [1928] and Parsons [1931]. Newburgh and Curtis [1928] found that protein in the form of dried liver was particularly harmful; on the other hand Newburgh and Marsh [1925], who observed renal lesions after intravenous injection of certain amino-acids, were unable to ascribe the toxic effect to any particular compound.

According to Drummond and his colleagues and Hartwell [1928] high protein diets decrease the growth-rate of rats, whilst the reverse was claimed by Osborne and Mendel [1924]; Slonaker [1931] differentiated between the unfavourable effect of high protein intake on activity, reproduction and life-span

[1] William Gibson Research Scholar.

[2] In the complete oxidation of 100 g. of average protein with N : P ratio 17 and N : S ratio 14·5, phosphoric acid is produced equivalent to 30 cc. *N* alkali and sulphuric acid equivalent to 50 cc. *N* alkali.

and its favourable effect on lactation, growth of the young and freedom from tumours.

The question arises whether the discrepancies among these findings can be explained, in part at least, by the differences in the acid-base balance of the diets used by the various workers. Thus, Osborne *et al.* [1926–27] supplied vitamin B partly in the form of alfalfa which would raise the allowance of fixed base. The evil effect of dried liver might be explained by its potential acidity, whereas the protective action of unheated liver could possibly be ascribed to one of the constituents of the vitamin B complex. It was considerations such as these which led to a re-investigation of the relationship of protein, acid-base balance and vitamin B with regard to impaired growth-rate and albuminuria. It is interesting to observe that Watson [1910] found softening of the bones of his experimental rats when he fed them on meat diets without any additional alkali. These changes suggest that, in the absence of basic substances from the diet, the calcium of the bones was mobilised for purposes of neutralisation. Presumably, carnivorous animals under natural conditions cover the acid-producing elements of their meat diets by eating bones.

The rôle of common dietary salt mixtures in relation to the acid-base mechanism had been touched upon by Hassan and Drummond [1927], who found that when the growth-rate of rats was retarded by diets containing a large proportion of protein, autoclaved marmite was protective, while the ash of marmite offered no protection against the inhibitory effect on growth. A special nephrotoxic action of cystine has been assumed by Cox *et al.* [1929] and others who have observed an acute toxic nephrosis to occur when cystine is added to the food of young rats. The addition of 0·1 g. cystine means a virtual increase of 1·6 cc. of *N* acid. The question of cystine nephrosis has formed the subject of another paper [Bell, 1933, 1].

We have been in the habit of thinking that the acid-base mechanism of most animals (the rabbit, among laboratory animals, being an exception) is extraordinarily efficient, and that ammonia can readily be utilised when fixed base is deficient. However, Farquharson *et al.* [1931] report that, whenever there is a rise in the ammonia excretion there is simultaneously an increased loss of calcium.

Experimental.

Experiments were planned to determine whether the following diets could be improved by the addition of a mixture of sodium citrate and potassium carbonate:

1. Diets containing 70 % caseinogen.
2. Egg-white diets: (*a*) containing 20 % egg-white, (*b*) containing 66 % egg-white.

1. Diets containing 70 % caseinogen.

A. Three dozen rats were used in the first series to ascertain whether the previous experiences of this laboratory could be confirmed, *i.e.* that rats ingesting large amounts of caseinogen exhibit subnormal growth, but that the inclusion of an additional ration of yeast overcomes this retardation of growth. Three groups of rats received the following diets respectively:

	X	Y	Z
"Light white casein"	20	70	70
Dried brewer's yeast	5	5	15
Rice starch	68	28	28
Cod-liver oil	2	2	2
Salt mixture (McCollum's)	5	5	5

The average growth curves (Fig. 1) demonstrated that the diet containing 70 % caseinogen with 5 % yeast had an adverse effect on growth, but that a decided improvement occurred with 15 % yeast.

B. Several groups of rats, carefully chosen litter-mates, received diets Y and Z as above, but were further subdivided so that half of them were given enough of a potentially alkaline mixture to reduce the acidity of their urine almost to zero. The mixture of salts contained sodium citrate (10 parts) and potassium carbonate (7 parts); excessive alkalinity was avoided for fear of damaging the kidneys thereby; the urine, therefore, was kept just acid. The graphs in Fig. 2 *a* show that no improvement occurred when the alkali was added; growth of rats ingesting diet Y was still subnormal, though, probably through some seasonal variation, this set of rats grew rather more vigorously than those of Fig. 1. It appears, therefore, that 5 % McCollum's salt mixture in the diet is adequate to meet the need for fixed base when the proportion of caseinogen in the food is 70 %.

Although the majority of the animals studied in this series gave us no reason to believe that the subnormal rate of growth encountered when the diet contained 70 % caseinogen could be attributed to the acidity, one anomalous result must be mentioned in which a number of rats, obtained from a dealer, behaved rather differently. Those being fed on diet Y without alkali grew more slowly than those consuming the extra mixture of potentially alkaline salts. But as this result could not be repeated, we were compelled to regard it as purely fortuitous.

Incidentally, the graphs in Fig. 2 *b* show that the addition of alkali to a 70 % caseinogen diet containing 15 % yeast does not enhance the growth still further; nor does it produce supernormal growth when compared with the rate of growth when caseinogen constitutes 20 % of the diet.

2. Egg-white diets.

Confirmation has been obtained of the observations of Bateman [1916], Boas [1924; 1927], and Parsons [1931] that uncoagulated egg-white in the food of rats rapidly causes diarrhoea which is fatal if the diet is not amended.

In the experience of Boas with 20 % egg-white, coagulated and dried in an oven, then added to the food, rats grew normally at first, then declined and died; against this adverse effect, certain varieties of starch were protective. In the following experiments, carbohydrate was therefore supplied in the form of cane sugar, as had been done by Parsons. Chinese egg-white was prepared in one of two ways—it was coagulated and finely minced; or coagulated, dried at 80° and finely ground. Using the egg-white prepared as above, diets were compounded of:

20 % egg-white (dry basis),
68 % cane sugar,
5 % dried brewer's yeast,
5 % McCollum's salt mixture,
2 % cod-liver oil.

The severe effect reported by Boas failed to occur. There was, however, diminished growth-rate compared with that of animals ingesting food containing 20 % caseinogen (Fig. 3). No improvement was induced by adding alkali to the food. Hence, the depressed growth rate resulting from diets containing 20 % egg-white cannot be attributed to the acidogenic properties of the cystine in which it is rich.

We also repeated the experiments of Parsons, using 66 % egg-white (dry basis), with and without potential alkali, and found that no amelioration took

place when the diet included the extra base (Fig. 4). The finding that 5 % McCollum's salt mixture was adequate to cover the acid produced by the meta-

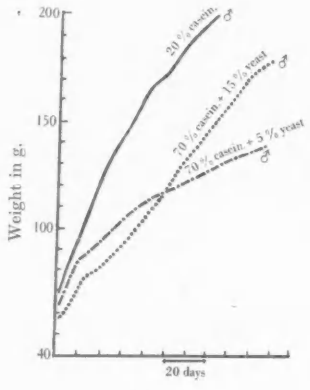

Fig. 1.

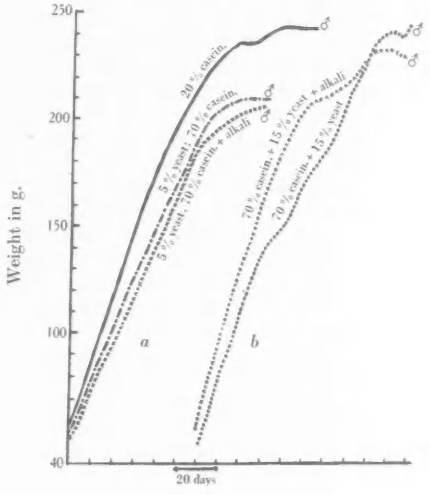

Fig. 2.

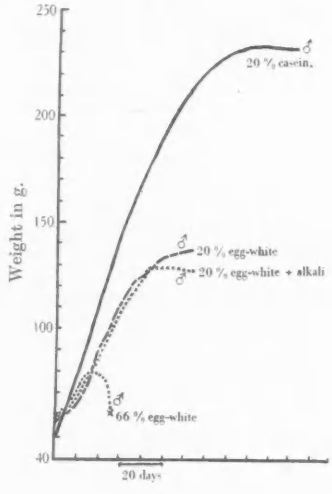

Fig. 3.

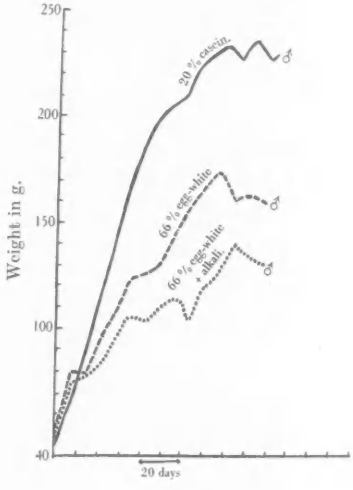

Fig. 4.

bolism of 66 % egg-white in the food thus dismissed the original idea of the research. What follows, therefore, is a digression in which egg-white diets are discussed.

Not only did we use egg-white, coagulated and dried at 80°, according to Parsons' method of preparation, but also egg-white coagulated and minced. Both Chinese and fresh egg-albumin gave, as Parsons found, comparable results. Care was taken not to overheat the egg-white. The food consisted of:

66 % egg-albumin (dry basis),
22 % cane sugar,
5 % salt mixture (McCollum's),
5 % dried brewer's yeast,
2 % cod-liver oil.

With our experiments, one group of rats exhibited considerable resistance to the diet; this group, charted in Fig. 4, failed to develop the acute nephritis which Parsons had observed; they grew comparatively well, and most of this group did not get the pellagra-like condition which she described. Two individual rats, however, which merely maintained their weight at about 60–80 g. for twelve weeks, were affected with the scaly and gangrenous condition of the tail, which has now been observed on a variety of diets, and which, in this laboratory, we are inclined to attribute to chronic malnutrition arising from the absence of one of the vitamin B factors.

Another set of animals, however, also receiving the 66 % egg-white diet, manifested at an early stage the symptoms of acute nephritis as described by Parsons, and most of them died (Fig. 3). The urinary findings included albuminuria, renal cells, epithelial casts, red blood cells; and in addition determinations of the blood-urea gave the high average figure of 131 mg. per 100 cc. blood. Consistently with this, the "clinical" symptoms were drowsiness and anuria, while the kidney sections showed acute nephritis. But the feature which we thought significant about the results was that one or two animals recovered without any alteration in the diet—a fact already observed by Parsons in the case of egg-white diets; similar recovery has also been reported by Cox, Smythe and Fishback in the case of cystine nephrosis. The kidney symptoms of such rats as did not die cleared up, and the animals continued to maintain their weight. The idea became insistent that the probable explanation of the train of events was that a septicaemia had occurred; that the kidneys had been incidentally affected; and that when an immunity had been established, recovery took place. An attempt was made to determine whether there was a blood infection; seven of the affected animals were killed with chloroform, and blood cultures were made, five of which were sterile, one grew a streptococcus, another grew a coliform bacillus. The results were therefore inconclusive. A possible explanation of this hypothetical septicaemia was sought in the altered conditions in the intestine, as a breeding ground for bacteria. It will be seen that the animals which were doing comparatively well on the 66 % egg-white diets exhibited irregularities in their weight curves (Fig. 4); and moreover we observed that they were subject to slight, intermittent diarrhoea. The possibility suggested itself that, for some unexplained reason, egg-white was perhaps less readily digested than other proteins, and that this might make for a greater percentage of protein degradation products in the intestine. It became interesting, therefore, to determine the percentage of nitrogen in the faeces, and it was found that excreta collected just before the rats developed nephritis contained approximately 0·06 g. N per g., whereas animals eating the stock diet were excreting 0·02 g. N per g. faeces. Later, in animals which had recovered, the quantity of nitrogen being excreted from the intestine became less—*viz.* 0·03 g., presumably as the

intestine responded by increased activity. Similar estimations of nitrogen on the excreta of rats ingesting 70 % caseinogen gave the average figure of 0·03 g.

With the presence of excessive nitrogenous material in the gut, there was the likelihood of increased numbers of putrefactive organisms in the intestine. The urine was found to give a highly positive test for indican as compared with the urine of rats being fed on the 70 % caseinogen diet. Even the rats which were doing comparatively well on the egg-white mixture always gave a strongly positive test for indican. Possibly, this gives a clue to the severity of the results of an egg-white diet as compared with other high-protein diets. The underlying effect is perhaps the presence of substances like cystine which alter the oxidation-reduction potential of the gut, and so favour the growth of specific types of organisms. Work on *in vitro* cultivation of anaerobes by Lepper and Martin [1929] has demonstrated that, for example, the addition of cooked muscle to culture medium favours the growth of anaerobes. It seemed reasonable to infer that the severe diarrhoea attendant on the administration of uncoagulated egg-albumin was only an exaggeration of this state of affairs; that since raw egg-white is not readily acted on by pepsin-HCl, as Bateman observed, there is an increased chance for bacterial multiplication in the intestine. Feldman [1920] quotes Dementjeff as stating that giving the raw whites of two eggs to a baby resulted in the appearance of indican in the urine. Recently, Nedzel and Arnold [1930–31] have shown that in the presence of uncoagulated egg-white, the permeability of the intestine to *B. prodigiosus* is increased.

Urinary examinations.

During the course of these feeding experiments, the rats were put into metabolism cages for the purpose of testing their urines for "total acidity" and for albumin. It was observed from estimations of acidity and ammonia that the rat is well able to utilise ammonia as a base. It was also obvious that considerable amounts of calcium could be absorbed into the blood stream and excreted by the kidneys; this was true whether the calcium was given as lactate or carbonate. Thus, when some stock rats were being compared with animals on the experimental diets, it was found that calcium carbonate became deposited on the funnel and urine trap, and the urine, even in the presence of toluene, became alkaline on standing. Presumably, this calcium was excreted as the bicarbonate. That rabbits can absorb calcium given as carbonate has been demonstrated by McGowan *et al.* [1931], who incorporated it in the diet and found that the rabbit's urine after standing contained a deposit of calcium carbonate. This ability of the rat and of the rabbit to absorb calcium given as carbonate from the alimentary tract seems worth mentioning in view of the belief commonly held that human beings have difficulty in absorbing calcium.

Tests for albumin in the urine revealed the fact that mature male rats normally excrete about 0·5–0·8 g. albumin per litre of urine, but that the urine of females is generally free from albumin. The results of a study on this point have been published in a separate note [Bell, 1933, 2]. Females ingesting the diets which contained a large proportion of caseinogen excreted no albumin; and no increase occurred under these conditions in the amount excreted by the males. From urinary examinations no evidence of kidney derangement was obtained.

Estimation of blood-urea.

After the animals had been fed for six months on the diet containing 70 % caseinogen, their fasting blood-urea was determined; they were fasting for six hours before being killed with chloroform. The average blood-urea for the

group receiving additional alkali was 39·9 mg. per 100 cc.; for those without alkali, the average was 41 mg.; for a control group of whose food the protein constituted 20 %, the average was 42 mg. Blood analyses by Jackson [1924–25], and by Osborne *et al.* [1926–27] showed that rats receiving high protein diets had a higher level of non-protein-nitrogen and of urea than rats ingesting normal amounts of protein; their animals were presumably not fasting. Addis, MacKay and MacKay [1926–27] have already demonstrated that the non-protein-nitrogen level quickly falls to normal when food is withheld; this extra nitrogen appears to be due, therefore, to the presence of large amounts following absorption rather than to the inability of the kidney to excrete it.

Histology.

Examination of the kidneys of rats which had received food containing 70 % caseinogen for six months failed to reveal any pathological changes. According to Newburgh, it requires a longer period than six months' feeding with excessive caseinogen to produce degenerative changes.

Summary.

1. The experience of those workers is confirmed who find that rats exhibit a subnormal rate of growth when fed on diets consisting largely of caseinogen and containing the usual proportion of yeast serving as the source of the vitamin B complex, and who also find that the inclusion of further proportions of yeast supplement renders the high-caseinogen diet adequate for growth.

2. The addition of potential alkali to diets containing a large percentage of caseinogen or coagulated egg-white does not improve the growth-rate. The presence of 5 % McCollum's salt mixture in the diet is therefore sufficient to meet the need for fixed base when high protein diets are given.

3. No evidence of kidney damage has been obtained in rats which have been fed for six months on diets containing 70 % caseinogen.

4. The presence of uncoagulated egg-white in the diet of rats is rapidly fatal, as has been found by Bateman and others.

5. Subnormal growth occurred as a result of feeding rats with diets containing 20 % coagulated egg-white, but the decline in weight reported by Boas failed to occur.

6. Subnormal growth also resulted when the food included 66 % egg-white; some of the animals developed acute nephritis and died. The findings of Parsons are therefore confirmed.

7. Examination of the faeces for nitrogen and of the urine for indican in the case of animals receiving egg-white diets led to the belief that excessive putrefaction in the intestine occurs when these diets are given.

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CXCV. CONDITIONS OF HYPERTROPHY OF THE SEMINAL VESICLES IN RATS.

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PART I.

Voss and Loewe [1931] and Moore and Gallagher [1930] have described certain restoration phenomena in secondary sex organs of castrated mice and rats respectively, by treatment with male hormone, the former authors being the first to use changes in the epithelium of the seminal vesicles as a rapid cytological test for the action of the hormone. Complete restoration was claimed by Moore and Gallagher [1930] to result from administration of 5 capon units (as defined by Gallagher and Koch [1930]) daily for 21 days, this daily dose being equivalent to 7·5 capon units as defined by Freud *et al.* [1932].

As will appear from the work to be described below, the male hormone is only one of the hormones which act on the seminal vesicles, of which it causes the epithelium to hypertrophy; the complete restoration claimed by the above mentioned authors is impossible with male hormone alone and was probably obtained by the use of preparations contaminated with oestrin or its homologues, these substances alone being stimulants of smooth muscular hypertrophy.

In this laboratory particular attention has been paid to parallel experiments on capons and on rats. The reaction of capons to preparations of differing purity and obtained from various sources proved to be reliable in our hands, contrary to statements of Korenchevsky *et al.* [1933], who reject the capon method as unreliable, and advocate testing on rats. To their statement the same criticism applies as in the case of Moore and Gallagher [1932].

In capons general conditions such as sunlight, health of the animals and, according to de Fremery [1931], thyroid function during various seasons as well as the male hormone influence comb growth. It must be especially pointed out in this connection that ketohydroxyoestrin (menformon) in crystalline form up to 1000 m.u. a day has no influence whatsoever upon comb growth. The seminal vesicles on the other hand are distinctly influenced by menformon or its homologues and as will be shown in Part II, these substances may be important in facilitating the action of male hormone at comparatively low doses of the latter.

On account of the irregular response of the seminal vesicles to various preparations, it was impossible to establish a constant ratio of minimum active quantities of male hormone between rats and capons. Continued fractionation of extracts led to their gradual loss of potency with reference to seminal vesicles without corresponding loss of potency in capons. The preliminary assumption of a separate comb growth and seminal vesicle hormone was soon replaced by another conception, which will be presented below.

Choice of material.

Seminal vesicles are easy to prepare and they respond rapidly to castration or treatment. They may serve as indicators of the hormonic state of the genital system, although there are other organs, such as the preputial glands of rats, the reaction of which is not conditioned by the presence of some other active substance though it is less specific than that of seminal vesicles. With these organs we propose to deal later.

The weight of the seminal vesicles of adult rats ranges between 120 and 800 mg. varying with (1) secretory state, (2) size of testicle, (3) structure of testicle, (4) size of hypophysis, thyroid and adrenals, (5) general condition of the animal. After castration of adult rats the weight of the seminal vesicles varies from 90 to 350 mg. The atrophy does not reach a definite basic point even after months. There is a marked difference between rats castrated during infancy or later; in the former the seminal vesicles cease to grow at once after removal of the testicles. Whilst a complete restitution of the comb and other male characters was obtained in capons by continued treatment with male hormone [Freud *et al.*, 1932] it is difficult for male hormone to act on completely atrophic seminal vesicles, just as progestin [Corner and Allen, 1929] is potent only after preparation of the muscular tissue of the uterus of infantile rabbits by oestrin. The seminal vesicles of rats castrated during infancy may be taken therefore as indicators, not of male hormone only, but of complete testicular activity. This idea is supported by experiments on senile and on non-castrated infantile male rats with gonadotropic substances, which, stimulating the whole of the testicle, lead to an almost complete (precocious) development of the seminal vesicles [de Jongh, 1930].

On these grounds we decided to use infantile rats as test material and simplified the method by beginning their treatment almost immediately after castration, instead of awaiting maturity. In these animals we aimed at forcing a precocious development of secondary sex characters. Under these circumstances the weight of the seminal vesicles is the first indicator of the effect of a certain treatment, the histological picture being used for the analysis of their growth.

Technique.

25–45 g. (3–5 weeks old) rats are castrated by the transscrotal route. There is no bleeding; one stitch closes the wound. This operation has the object of preventing spontaneous genital development during the experiment. Three days later a course of 8 injections is started, litter-mates as far as possible being equally distributed between experimental and control groups. Twice a day 0·1 cc. liquid is administered subcutaneously. On the day following the last injection the animals are killed with chloroform. Both seminal vesicles are cut out in one semilunar piece and weighed on a torsion balance immediately after removal. A narrow bridge of prostate tissue connects the two lobes. Formaldehyde is used for fixing and haematoxylin-eosin for staining.

Histology of seminal vesicles.

Seminal vesicles have a peritoneal covering, connective tissue stroma with smooth muscle tissue imbedded therein, a small zone of subepithelial stroma and a single layer of cylindrical epithelial cells lining a sinuous cavity. In moderate hypertrophy the sinuosities are macroscopically visible.

Hypertrophy. Muscle cells large, with bright nuclei. Frequent mitoses. Epithelium high cylindrical, with basal nuclei reaching to 1/3 or 2/3 of the total height of the cells and leaving

a protoplasmic margin free towards the cavity. Mitotic cell divisions characterise the beginning of hypertrophy, they diminish later on. The cavity contains basophilic, homogeneous secretion.

Atrophy. Small muscle cells with dark nuclei, low epithelium of cubical cell form, without protoplasmic margin, the nuclei filling the cell bodies almost completely. No mitoses, only sporadic pycnosis of nuclei, imitating cell division.

Muscular hypertrophy does occur without epithelial development, while epithelial hypertrophy without a certain muscular development was never observed.

Apparent paradoxical effect of menformon.

In a previous paper [Freud *et al.*, 1933] muscular hypertrophy of seminal vesicles under the influence of menformon (ketohydroxyoestrin) was described under this heading. Male and female hormones are produced in both sexes in approximately equal quantities, as judged by their excretion in the urine of males and non-pregnant females. The two substances are distinctly different pharmacologically, they act on different tissues. The effect of menformon in males is only apparently paradoxical.

Weight of seminal vesicles.

In Table I 32 untreated castrated male animals are classified according to body weight and weight of the seminal vesicles.

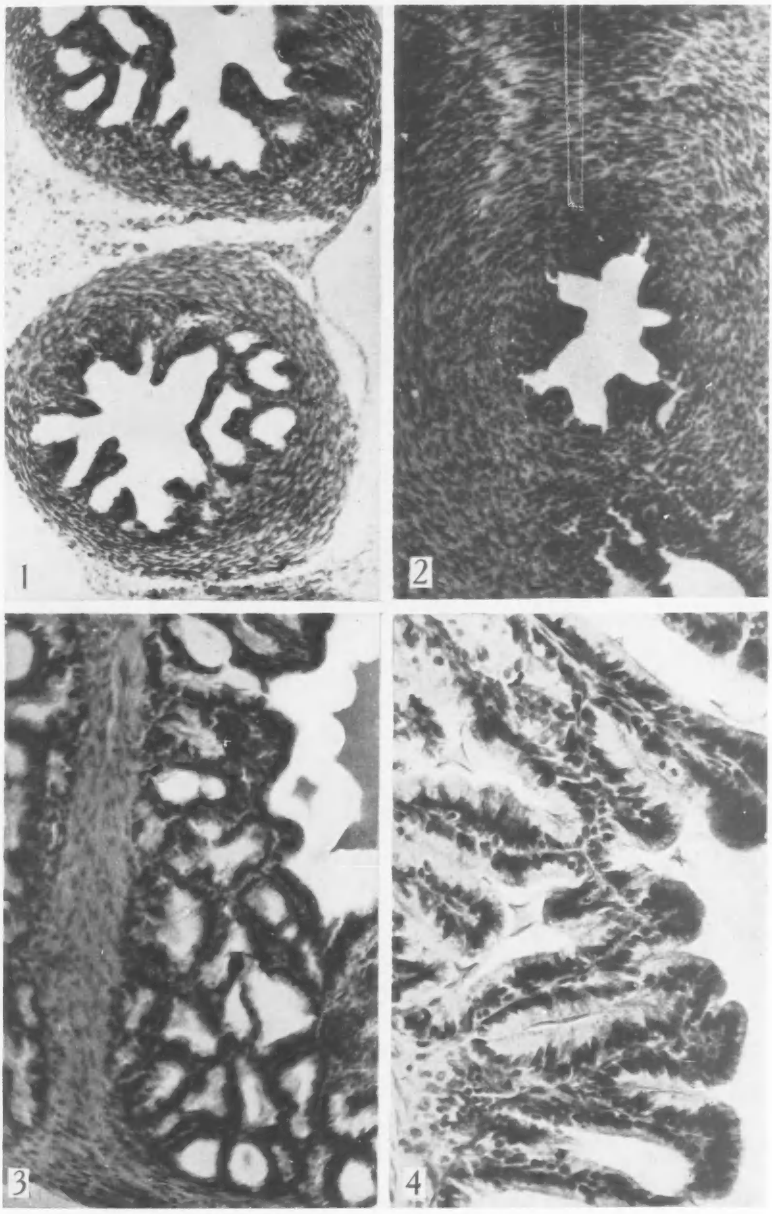
Table I.

Body weight g.	Weight of seminal vesicles (mg.) 3–4	4–5	5–6	6–7	7–8	8–9	9–10	Total
21–25	—	2 (Feb.)	1 (Feb.)	—	—	—	—	3
26–30	1 (May)	1 (July)	—	—	—	1 (July)	—	3
31–35	—	—	—	1 (July)	2 (July)	—	—	3
36–40	1 (July)	1 (May)	1 (Jan.)	1 (Mar.)	2 (Jan.)	—	—	
			1 (May)	2 (May)	2 (Feb.)	1 (May)	1 (Feb.)	13
41–45	—	—	—	1 (Feb.)	1 (Feb.)			
				1 (July)	1 (May)	—	—	4
46–50	—	—	—	—	1 (Feb.)	1 (Mar.)		
						1 (July)	—	3
51–55	—	1 (Feb.)	—	—	1 (Mar.)	—	1 (May)	3
Total	2	5	3	6	10	4	2	32

From this table we may conclude that seminal vesicles weighing more than 10 mg. scarcely ever occur in animals of less than 55 g. Wallen-Lawrence and van Dyke [1931] state that infantile non-castrated rats below 40 g. body weight have seminal vesicles of an average weight of 9·24 mg. Our material with reference to non-castrated rats confirms this statement. Geographical and racial differences do not seem to have any noticeable influence. The seminal vesicles of castrated and of intact infantile males between 25 and 45 g. body weight practically do not differ. Histologically no large difference exists either, with the possible exception that nuclear pycnosis occurs in castrates only.

How does male hormone act?

Assuming that the histological state of the epithelium of the seminal vesicles decides whether a male hormone preparation is potent or not, the question of the relation between weight and histology arises. The degree of epithelial response may be classified according to two criteria: height of epithelium and number of mitoses. In this paper however the response is considered as a whole without further differentiation. A response is positive whenever the protoplasmic margin exceeds one-third of the cell height or when at least one mitosis is discovered per visual field at a moderate magnification.



The photomicrographs represent seminal vesicles of infantile castrated male rats. 1, 2 and 3 all at the same low magnification.

Fig. 1. Without treatment; atrophic smooth muscle and epithelium.
Fig. 2. Treatment with menformon; hypertrophic smooth muscle, atrophic epithelium.
Fig. 3. Treatment with male hormone combined with menformon; totally hypertrophic seminal vesicle with ramified lumen and secretion.
Fig. 4. The same preparation as Fig. 3 at higher magnification, showing details of hypertrophic epithelium (note mitoses).

In Table II 940 animals are classified according to weight of seminal vesicles and positive or negative response of the epithelium. From this we see that epithelial responses become positive between 16 and 19 mg., while above 20 mg. with one exception, no negative response occurs. Positive responses below 14 mg. are exceptional. From this table it appears reasonable to assume 19 mg. as the *positivity limit*.

Table II.

Weight of seminal vesicles in mg.	Epithelial response		Percentage		Number of animals
	Positive	Negative	Positive	Negative	
4–5	0	16	0	100	16
6–7	0	36	0	100	36
8–9	1	68	0·15	99·85	69
10–11	3	109	2·7	97·3	112
12–13	9	125	6·7	93·3	134
14–15	26	121	17	83	147
16	13	56			
17	21 34	38 94	27	73	128
18	28	17			
19	32 60	3 20	75	25	80
20–21	69	0	100	0	69
22–23	37	1	98	2	38
24–25	27	0	100	0	27
26–27	17	0	100	0	17
28–29	14	0	100	0	14
30–31	7	0	100	0	7
32–33	4	0	100	0	4
34–35	4	0	100	0	4
36–37	4	0	100	0	4
38–39	3	0	100	0	3
40 and more	31	0	100	0	31
Total	350	590	—	—	940

Sixty-four animals are not classified in Table II, because the treatment of these marks them as a special group. Their epithelial response was consistently negative, although the weights of their seminal vesicles were between 14 and 30 mg. They were treated with one of the following kinds of preparations: (1) unpurified or crystalline oestrin (menformon); (2) crystalline menformon *plus* subthreshold doses of male hormone; (3) oestrin fractions of male urine after separation from male hormone.

Table III shows the distribution of the cases according to the weight of the seminal vesicles.

Table III.

Weight of the seminal vesicles in mg.	Number of (negative) epithelial responses
14–15	1
16–17	4
18–19	28
20–21	13
22–23	9
24–25	6
26–27	2
28–29	0
30	1
Total	64

From Tables II and III, the following conclusion seems justified. In low doses male hormone is inert. In comparatively high doses, in seminal vesicles of above 14 mg. weight, the frequency of "positive" epithelial responses increases

rapidly until above 19 mg. practically all the reactions are positive. However, there are doses of male hormone insufficient to produce epithelial changes but just sufficient to help oestrin in producing muscular hypertrophy. Hence in seminal vesicles between 14–30 mg. histological control is advisable to detect whether the effect is merely due to oestrin and subthreshold doses of male hormone, or whether the dose of male hormone was sufficient to cause epithelial hypertrophy. Of course not all animals respond to threshold doses.

Reliability of the reaction.

Three criteria are decisive with reference to this question.

(1) *Individual response variations within one group of experimental animals.* This criterion is in favour of reliability in a large majority of the seminal vesicle reactions.

(2) *Increasing response to increasing doses.* Only those experiments are to be used for comparison which were carried out at the same time, so as to exclude group variations due to circumstances changing with time. 126 serial experiments with two or more doses and with exclusion of all those cases where all doses were subthreshold yielded the following result:

	Times	Percentage
Higher dose—increased response	87	69·05
Different doses—equal response	24	19·05
Paradoxical result, higher dose, weaker response	15	11·9

The doses varied as 1:2:4:8 and so on. A group response of otherwise comparable animals was considered different when weight differences of the seminal vesicles averaged more than 2–3 mg., or when histology gave the impression of higher intensity of response.

(3) *Reproducibility of results.* This criterion includes the time factor of response. 57 times threshold doses of the same fractions of a preparation were administered repeatedly to groups of rats during different weeks. 21 experiments were discrepant, 36 yielded identical results. Of these latter however both experiments were negative 16 times and so of doubtful value in this connection. Our last criterion is thus unsatisfied, at least as long as the exact amounts of oestrin contaminating our preparations are unknown.

Influence of body weight and growth.

In Table IV the influence of body weight upon the growth of seminal vesicles is indicated. This table is constructed as follows: 3 animals of one experiment are classified as "light," "medium" and "heavy" in the corresponding horizontal

Table IV.

	Weight of seminal vesicles			
Weight of animals	Light	Medium	Heavy	
Light	235	95	107	
Medium	109	109	88	
Heavy	107	75	264	Total: 1189

lines of the table, in the columns according to the weight of their respective seminal vesicles. Every animal is placed by comparison with the two others of the same experiment. When two animals of a group have equal weights, they are placed in the "light" and "heavy" rows in the columns corresponding to their respective seminal vesicles.

Table IV refers to 1189 animals, treated with potent preparations, showing a marked tendency to respond better with increasing weight. By considering the diagonal of this table from left above to right below as the median line of normal reactions and taking the sum of the figures along it and along the lines parallel with it, the following frequency range results: 107, 184, 608, 183, 107. This is a normal or a Gauss frequency curve, in which the summit is along the diagonal connecting those values which show that larger animals tend to react more strongly than small ones.

Similar conclusions may be reached by considering the influence of growth of the animal during the experiment upon growth of the seminal vesicles. This is shown in Table V.

Table V.

	Weight of seminal vesicles			
Growth of animals	Light	Medium	Heavy	
Small	221	84	148	
Medium	81	81	84	
Great	143	99	220	Total: 1161

Frequency distribution along diagonals: 143, 180, 522, 168, 148.

From these figures we see a tendency for the seminal vesicles to grow most in animals which themselves grow most.

The conditions for optimum growth of seminal vesicles are present in comparatively large and actively growing animals. By introducing body growth as essentially affecting seminal vesicle response to treatment, the existence of a number of external and internal factors is revealed in these experiments. These again account for the extraordinary variability of the reaction and for the difficulty of reproducing threshold reactions at different times. The influence of hypophyseal (growth?) factors upon this reaction will be discussed in another paper. The necessity of a large number of experiments preceding any kind of consistent conclusion is explained by these circumstances.

PART II.

This part deals with the experimental determination of the rôle of the follicular hormone (oestrin) in the development of seminal vesicles.

Varying potency of capon units of male hormone in rats.

Forty-one preparations and fractions of male hormone, extracted from testicles and from male urine, were tested on capons and on rats. In Table VI the comparative values of potency in these two kinds of animals are noted. Standardisation could not be done completely in all cases, the probable minimum active quantity is in many cases more or less than the one noted in the table, both in rats and in capons. Yet from the figures it is apparent that a more exact determination would have made the irregularity of response of rats in most cases still more marked.

A large number of preparations, obtained by the same procedures as those noted in Table VI, were tested on rats only. While the quantity of male hormone in such preparations, whether obtained from urine or from testes, when determined by the capon test, became with increasing experience almost constant,

Table VI.

Prep. from testis	g. tissue containing 1 capon unit	g. tissue containing 1 rat unit	1 capon unit equal to rat units	Prep. from urine	Litres urine containing 1 capon unit	Litres urine containing 1 rat unit	1 capon unit equal to rat units
107	100	100	1·0	120 (II)	2·0	0·4	<0·2
112	20	10	0·5	139 (I)	0·5	>0·5	>1·0
113	50	150	3·0	149 (I)	0·5	0·8	1·6
114	100	>20	>0·25	178 (I)	0·5	0·4	?1·0
131	80	20	0·2	178 (II)	5·0	>1·0	?0·2
141	50	15	0·3	294 (II)	1·0	>1·0	>1·0
143	50	20	0·4	315	2·0	2·0	1·0
147	25	40	1·6	315 (II)	2·0	>4·0	>2·0
167	<?50	50	>1·0	Z 12	<0·25	<2·0	?0·1
273	50	>50	>1·0	Z 15	2·0	0·1	0·05
Z 4	50	25	0·5	Z 16 (I)	1·0	>0·4	>0·4
Z 7	25	25	>1·0	Z 22	0·2	0·2	1·0
Z 13	25	>40	>1·6	Z 26 (I)	3·0	⩾0·4	0·14
338	50	80	1·6	Z 26 (II)	3·0	>0·4	>0·14
358	50	>50	>1·0	390	2·0	>0·4	>0·2
Ho	50	80	1·6	390 (II)	2·0	>0·4	⩾0·2
470	100	⩾100	>1·0	390 (II)	2·0	>0·4	⩾?0·2
491	50	>50	>1·0	455	0·5	>0·4	>0·8
510	50	>50	>1·0	455 (II)	0·5	0·2	>0·4
				459	0·5	>0·2	>0·4
				459 (II)	0·5	>0·2	>0·4
				514	0·5	0·8	?1·6

the potency of such preparations in rats remained very variable; hence the chemical procedure applied for the preparation of male hormone active in birds must be considered inadequate for obtaining a product, which is fully active in rats. (According to recent results, by improved methods the number of bird units found in testes is about 60 units per kg.; in urine about 30 units per litre.)

Pace-making.

In previous sections the importance of ketohydroxyoestrin for seminal vesicles has been emphasised. Besides this form of oestrogenic material, other substances have been described by Marrian [1930], Butenandt and Marrian [1931], Doisy and Thayer [1931], Girard *et al.* [1932], de Jongh *et al.* [1931] and others, which are chemically related to the original follicular hormone (menformon), but have different oestrogenic potencies. The procedure of purification of male hormone includes the elimination of these substances as well as of menformon. Quantitative data with reference to the biological potency of these substances are scanty, owing to their incompletely defined biological effects. The experience gathered in experiments such as are noted in Table VI led to systematic study of the influence of elimination of oestrogenic material and homologues from purified male hormone preparations. It was ascertained that their loss of potency was related somehow to their freedom from oestrogenic material. Preparations, which as crude extracts were potent both in rats and in capons, became inactive in rats in both fractions after separation of the male from the follicular hormone, while the fraction containing the male substance when tested in capons, proved to be almost as potent as the crude extract. This observation led us to the attempt to recover the original activity in rats by recombining the two isolated fractions, after some purification of both in the separated state. This procedure was successful. In Table VII an example is given. The fraction used was the same as referred to already in Table VI under Z 26.

Table VII.

Fraction of urine extract	Daily dose in litres of urine	mg. seminal vesicles found in rat			Histology of epithelium
		I	II	III	
I (male hormone)	0·1	9	9	—	—
	0·2	10	10	—	Negative
	0·4	12	7	9	—
II (follicular hormone)	0·1	14	11	14	—
	0·2	11	16	—	Negative
	0·4	—	19	16	—
III (I+II)	0·1	23	13	—	—
	0·2	24	20	—	Negative
	0·4	27	25	—	—

From this table we see a considerable weight increase of the seminal vesicles after recombining the two fractions of the same preparation. The epithelium of these organs was "negative," while the smooth muscular tissue of group III in all cases yielded the picture of an intensive menformon effect. From Table VI it can be seen that Z 26 was a very weak preparation as far as male hormone is concerned and compared with other extracts of urine. (In Table VI the preparations marked with II are oestrin fractions, these being almost inactive in capons.) Apparently a small amount of male hormone, insufficient to affect the epithelium, may reinforce considerably the effect of oestrin upon the smooth muscle tissue of the seminal vesicles.

In Table VIII a case of combination of male hormone extracted from testicles with a menformon fraction of urinary extract is presented. (The latter is the same as in Table VII.)

Table VIII. *Preparation 338 and Z 26 (II).*

Capon units daily of 338	Urine litres equivalent daily of Z 26 (II)	mg. seminal vesicle found in rat		
		I	II	III
0·2	—	17	9	7
0·4	—	17	14	12
0·8	—	14	17	10
0·2	0·1	20	25	21
0·4	0·2	24	25	28
0·8	0·4	30	34	40

This experiment was done in July 1931, when we still thought that seminal vesicles weighing more than 19 mg. have always a "positive" epithelium. Later a few of these organs were investigated again. It was found that the one weighing 30 mg. (see Table VIII) also had "negative" epithelium. Again male hormone of relatively low potency reinforced the effect of oestrin upon smooth muscle tissue. The final proof that the effect of male hormone was also benefited by the combination with menformon, was obtained from experiments where male hormone was combined with crystalline menformon. These experiments are presented in Table IX. In this Table, whenever histological investigation was done, the negative (−) or positive (+) epithelial response is noted.

In Table IX only 27 parallel experiments on 3–12 animals each are presented, while 11 preparations of male hormone of varying purity were tested. These preparations were combined with doses of crystalline menformon varying from 6 to 100 units a day. A large number of similar experiments done later confirm these results.

Table IX.

Preparation of male hormone	Capon units daily	Average weight seminal vesicles in mg.	Histol. of majority of cases	Additional menformon M.U. daily	Average weight seminal vesicles in mg.	Histol.
153	0·75	7·8	–	6	7·5	–
167	2·0	29	+	6	24	–
338	0·4	12	.	10	12	–
167	1·0	20	+	15	15	–
514	0·8	12	–	20	11	–
149 I	0·2	7·5	–	30	8	–
167	0·2	11	–	30	16	+
358	0·25	10	–	40	21	–
358	0·5	14	–	40	20	.
338	0·4	10	–	50	17	+
358	0·25	11	–	50	17	–
147	0·4	17	–	60	22	+
358	0·25	11	–	60	28	.
358	0·5	14	–	60	22	.
470	1·0	6	–	60	15	–
470	0·8	6	–	60	13	–
491	1·0	19	.	60	20	+
491	0·75	10	–	60	15	–
455	0·2	10	–	60	15	–
459	0·17	10	–	60	19	+
459	0·2	9	–	60	14	?+
459	0·4	7	–	60	16	+
459	0·8	9	–	60	16	+
459	1·6	17	+	60	26	+
338	0·2	11	–	100	14	.
338	0·4	12	–	100	23·5	+
338	0·8	14	.	100	24	.

Less than 40 M.U. of menformon seem to have no effect when combined with male hormone. 60 units a day regularly reinforce the seminal vesicle reaction (of another 30 similar experiments 23 showed this phenomenon). The number of positive epithelial responses also increases, though less regularly. 0·4 capon unit of male hormone without oestrin hardly ever produces seminal vesicles of more than 14 mg. in 5 days, while 1·6 units a day are a dose, which with some regularity yields appreciable growth of the seminal vesicles without the aid of oestrin. In the majority of cases 0·4 unit, reinforced by 60 units of menformon, is sufficient to produce the weight of seminal vesicles at which positive epithelial response seems to be possible. From these data we may conclude that menformon increases the effect of male hormone about 4 times. A simple addition could not explain this increased reaction, first because menformon has no effect upon the epithelium of seminal vesicles even in much larger doses than those here applied and further because 60–200 M.U. of menformon alone tested in 24 animals yielded only exceptionally seminal vesicles of more than 11·6 mg. in 5 days.

Pace-making may thus be characterised as follows: our male hormone preparations, having a very much reduced content of menformon (as tested in castrated mice) act together with crystalline menformon upon the smooth muscle tissue of seminal vesicles. The two substances mutually reinforce each other's activity in this respect. Epithelium of seminal vesicles is influenced only by male hormone in a positive sense. This effect seems to depend on many accessory conditions. One of these is a certain dimension of the non-epithelial tissues of the seminal vesicles, seeing that according to our experience the frequency of positive epithelial responses increases rapidly in seminal vesicles of more than

19 mg. weight. The considerable number of cases in which (at least in infantile rats), in spite of comparatively large seminal vesicles, no positive epithelial response occurred proves that the size of seminal vesicles and a certain amount of male hormone are not yet always sufficient for a positive epithelial response, therefore this cannot be considered as a reliable test for male hormone as long as the rôle of other effective agents is not elucidated.

Besides menformon trihydroxyoestrin (Marrian) (crystals of Marrian) was tested in pace-maker experiments. Two preparations of male hormone (338 and 358) were used in these experiments. Their effect, when given alone, may be seen in Table IX. Table X shows the results of these experiments.

Table X.

Preparation of male hormone	Capon units daily	γ-Trihydroxy-oestrin daily	Average weight in mg. of seminal vesicles	Histology
338	0·4	1	14	–
338	0·4	3	12	–
358	0·3	4	15	–
358	0·3	5	17	.
358	0·5	5	22	.
338	0·4	5	15	+
338	0·3	5	11	–
338	0·3	6	17	.
358	0·2	10	20	+
338	0·4	10	18	+
338	0·8	10	21	+
338	0·4	20	15	+
338	0·8	20	18	+

From these data the conclusion seems justified that trihydroxyoestrin, when combined with male hormone, reinforces seminal vesicle response to about the same extent as menformon (ketohydroxyoestrin). A contamination of the preparation of trihydroxyoestrin with menformon could not possibly account for this result, because both preparations were tested on castrated female mice and the oestrogenic potency of the trihydroxy preparation was so weak that 20γ did not correspond to more than 1·5γ menformon, *i.e.* about 15 units. This amount of menformon is too little for pace-making. The possibility of contamination of both with a very active third substance still deserves consideration.

For trihydroxyoestrin this is a new biological effect, which if confirmed might serve as a method of testing. The fact that related compounds with comparatively low oestrogenic potency have about the same pace-maker effect as menformon, might perhaps account for comparatively strong responses of seminal vesicles to crude testicular extracts, while the testicle is known to contain very little actual oestrogenic substance. It has hitherto been impossible to determine quantitatively the non-oestrogenic compounds in various organs on account of the already mentioned lack of an adequate biological method for testing them. Trihydroxyoestrin when injected alone has, in doses of 4–10γ daily, the same effect as similar doses of menformon, *i.e.* the seminal vesicles show a slight muscular hypertrophy, and their weight reaches in 5 days an average of 11·5 mg.

An attempt to analyse pace-making.

The following experiment was done in order to ascertain whether pace-making depends on simultaneous administration of the two hormones involved or not. 30 rats were castrated, 15 of these were treated during 6 days with 60 M.U. of menformon daily, 15 left without treatment. On the 7th day the first group of 15 was divided into three groups of 5, their treatment with

menformon was stopped, they were treated daily, as usual, with 0·2, 0·4 and 0·8 capon unit of male hormone respectively, while the group of 15 animals, which were hitherto without treatment, was also divided into three groups of 5, to which the same doses of male hormone as to the first three groups and additional 60 units of menformon daily were administered. On the 5th day of the second stage of this experiment the animals were killed. The result is shown in Table XI.

Table XI.

	Average weight in mg. of seminal vesicles after treatment	
Capon units daily	In 2 stages	Simultaneously with 2 hormones
0·2	11	15
0·4	12	17
0·8	17	19

Only one of these groups showed histologically positive epithelial response, *viz.* that treated simultaneously with menformon and with the highest dose of male hormone.

A treatment with menformon preceding that with male hormone does not yield the same strength of pace-maker effect as does the simultaneous treatment with both hormones.

Anomalous histological phenomena.

In 58 groups of 3 or more animals treated by mixtures of male and follicular hormones, the seminal vesicles exhibit a partial detachment of their epithelial lining from the wall of the organs. The rounded cells are conglomerated in the centre of the lumen. This result occurred only twice after exclusive treatment with male hormone extracted from testicles; it was observed 56 times in cases where male hormone was combined with crystalline or non-crystalline oestrin and with trihydroxyoestrin. By prolonging combined treatments the regularity of this occurrence is enhanced. The phenomenon hardly ever occurred where a "positive" epithelial response was obtained with male hormone. It is ascribed to an unbalanced influence of oestrin. Similar epithelial changes, not unlike tumours, were observed in rabbits' uteri after a treatment with several thousand units of follicular hormone for a few months. Lately de Jongh in our laboratory (unpublished results) observed not only epithelial degeneration, but also a metaplasia from columnar to stratified epithelium in the proximal part of the seminal vesicles of mice and rats after prolonged treatment with oestrin. This significant result will be extended and published in due course.

Discussion.

An optimum growth of seminal vesicles under artificial conditions is not to be obtained by treatment with one hormone only (*e.g.* the male hormone). The effect of this substance upon smooth muscle tissue is only moderate, its main point of attack being the columnar epithelium. Oestrin and its various homologues, of which only one is mentioned in this paper (trihydroxyoestrin), act upon smooth muscle tissue, while they either do not affect columnar epithelium or induce degeneration. The structures resulting from forced treatment with these substances are somewhat reminiscent of tumours and open a possible development of tumour pathology.

The combined effect of follicular and male hormones upon seminal vesicles is a mutual reinforcement of each other's actions. For this phenomenon the

expression "pace-making" is suggested. Complete restitution of seminal vesicles in animals castrated while they were immature and treated after they became adult was not obtained even by combined treatment. This fact indicates already that compounds should be sought amongst the homologues of oestrin, which are still more potent than the ones used. We surmise that one of the homologues of oestrin might be more potent with reference to smooth muscle hypertrophy than oestrin itself and might account for the higher potency of crude extracts of male hormone than of its purified forms. It is reasonable to assume that natural development of male secondary sex organs is governed by two types (male and follicular) of hormones. It is obvious that for the measurement of the oestrin homologue active towards seminal vesicles, its oestrogenic potency gives no reliable basis. Hence we are unable to say without analysis of the effect on seminal vesicles of castrates, how much of these compounds does occur in testicles. This conception inaugurates a new line of research on "male hormones." It is quite certain that experiments, such as *e.g.* those of Moore [1932], where "complete" restitution was obtained, the preparations used must have contained besides male hormone the active principle which is highly potent in producing muscular hypertrophy. The same applies to other muscular organs, such as the vas deferens, also studied by Moore and by others.

Under these circumstances there is no question of a standardisation of "male hormone" (under this name being understood the substance acting upon the comb of capons) in these experiments. The use of rats for such a purpose though not impossible, could not be recommended before these matters are settled by careful biological analysis.

A further conclusion from these results is that antagonism of sex hormones within the secondary sex organs certainly does not exist, at least between male and female hormones. They do not inhibit each other's effects, but on the contrary, in adequate doses, they support each other (pace-maker effect). The nocuous influence of an unbalanced treatment with one of these substances may be alleviated or even turned to advantage by administration in adequate doses of the other one, but in no way is this balancing based upon inhibition.

The therapeutic application of one of these substances may fail without simultaneous application of the other.

The successful application of combined male and female hormone preparations in the same proportion as mentioned above by van Cappellen [1933] in the treatment of prostate hypertrophy confirms the last statement from the side of clinical experience.

Summary.

1. Simultaneous experiments on capons and on infantile male rats showed loss of activity of male hormone after purification as regards rats, while activity was retained as regards capons.

2. Oestrin promotes smooth muscle development in the secondary sex organs of mammals, hence its "paradoxical" positive growth effect with reference to seminal vesicles is only apparent. Male hormone is active in promoting epithelial hypertrophy in the same organs.

3. In hypertrophied seminal vesicles histology decides between these two types of effect.

4. Experiments on infantile rats are reliable as regards individual response in one group of animals, less so with reference to dose-action ratio, while weekly response variations are rather considerable.

5. Body weight and growth during the experiments have a marked effect upon the response to potent preparations.

6. Male and follicular hormones support each other's effects upon seminal vesicles. This is called "pace-making."

7. Trihydroxyoestrin, though its oestrogenic potency is much less than that of ketohydroxyoestrin (menformon), is an effective pace-maker in the same doses (6–10γ) as menformon. Our knowledge about the quantities of these and homologous substances occurring in organs and tissues is scanty.

8. Abnormal histological responses of the seminal vesicles may occur after treatment of the animals with inadequate combinations of sexual hormones. The significance of these phenomena, reminiscent of tumours, remains unexplained.

9. In clinical practice, the application of these substances is advised on grounds of symptomatology and the knowledge of their pharmacological effects, rather than on so-called "hormone-balance," which does not give any idea about the mutual influence of these substances. Therapeutic application of combined male and female hormone preparations in the treatment of prostate hypertrophy has proved successful.

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CXCVI. ABSORPTION SPECTRA IN RELATION TO THE CONSTITUENTS OF FISH OILS.

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In comparison with other organic materials the vegetable fats and oils exhibit, as a rule, a high degree of transparency to ultra-violet rays. Thus in many instances, *e.g.* olive oil and groundnut oil, a thickness of several millimetres of oil is necessary for appreciable absorption to be shown, and, even so, little of a selective nature is exhibited. From this it follows that the glycerides of the common fatty acids, saturated and unsaturated, are relatively transparent. The same is true of mammalian- and fish-body oils which do not contain vitamin A.

In the case of fish-liver oils the greater part of the selective absorption is usually due to vitamin A, but in a number of cases, *e.g.* codling-liver oils [Lovern *et al.*, 1931] the gross absorption is so low, sometimes only recorded by the use of undiluted oil, that it becomes reasonable to conclude that the absorption shown by the glycerides of the polyethylenic acids occurring in nature is inconsiderable and exhibits but little selectivity.

In these circumstances it is somewhat surprising to find that some constituents of the total acids obtained by saponification of many fats, particularly fish-liver oils, exhibit relatively intense absorption of a highly selective nature, a long series of sharply defined narrow bands, about $10 m\mu$ wide, being regularly observed, together with a few inflexions and fainter bands which are not always seen. After allowing for the vitamin A content of the richer oils it is found that the "acid" curve invariably lies well above the "oil" curve, showing the absorption to be not only more selective, but also considerably more intense, than that of the glycerides and other esters in the oils.

Table I summarises the spectral absorption of the total acids from a number of fish-liver and fish-body oils. The last three in the table are interesting in that, although they are not liver oils, they contain appreciable quantities of vitamin A.

Before attempting to interpret the above data it is essential to recall the main facts concerning the spectral absorption of those constituents of natural fats which do not belong to the fatty acids, namely glycerol, cholesterol, ergosterol, vitamin A, vitamin D, certain higher alcohols, *e.g.* oleyl, batyl, selachyl, chimyl and in certain cases (shark, dog-fish) the hydrocarbon squalene, which, however, shows no selective absorption and may be dismissed here. Many oils also contain small quantities of coloured substances not unlike carotenoids [*cf.* Lovern and Morton, 1931]. Glycerol, although nearly always the predominant non-acid constituent, amounting to 4–5 % of the original oil, does not absorb

Table I. *Absorption maxima of total acids from saponification of fish oils.*

Maxima and inflexions (italics) are given in $m\mu$, with the $E^{1\%}_{1\,cm.}$ values immediately beneath. Thus $\frac{378}{0\cdot12}$ implies an absorption band with a maximum at 378 $m\mu$, and an $E^{1\%}_{1\,cm.}$ value (*i.e.* log I_0/I for a 1 cm. layer of 1 % solution) of 0·12.

Monk (angler-fish)-liver oil	—		(420	400	377)		347	328	315·5	301	—	281	269	260	234 $m\mu$
			0·2	0·3	0·4		1·5	2·0	9·0	10·0		68	82	74	116 $E^{1\%}_{1\,cm.}$
Haddock-liver oil	—	*445*	*423*	*400*	377	*362*	350	330	316·5	301	—	282	269	—	Tail-off
		0·032	0·038	0·052	0·075	0·13	0·225	0·285	0·63	0·72		1·32	1·55		22
Turbot-liver oil	*460*	*442*	*422*	402	378	*360*	350	333	317	*301*	*288*	282	270	—	Tail-off
	0·07	0·1	0·14	0·21	0·31	0·45	0·7	0·9	1·18	1·45	2·3	2·72	2·76		26
Pollack-liver oil	*460*	—	*422*	401	377·5	*360*	349	331	316·5	302	*290*	282	270	—	Tail-off
	0·017		0·027	0·055	0·1	0·11	0·22	0·27	0·55	0·67	1·1	2·4	2·7		28
Ling-liver oil	—	—	*422*	402	377	*360*	349	332	316·5	*301*	*294*	283	270	—	Tail-off
			0·1	0·14	0·2	0·25	0·42	0·55	0·76	0·97	1·4	2·2	2·3		24–25
Dab-liver oil	—	—	*423*	402	378	*360*	348	333	316·5	*302*	*290–5*	282	270	—	Tail-off
			0·02	0·07	0·12	0·16	0·28	0·72	0·97	1·2	1·7	2·36	2·4		18
Cod-liver oil	—	—	—	392	375	*360*	350	330	316	302	—	281	270	259	234
				1	1·5	2	14	15	80	85		210	250	205	200
Sturgeon peritoneal fat	—	—	—	400	377	*361*	348	331	316	301·5	—	282	269	259	Tail-off
				0·025	0·95	0·065	0·15	0·2	0·31	0·35		0·93	1·2	1·25	22–23
Sturgeon pancreatic fat	—	—	—	*400*	377	*359*	348	332	317	*300*	—	282	270	*259*	Tail-off
				0·042	0·08	0·12	0·21	0·3	0·55	0·65		1·1	1·14	1·1	18–20
Conger eel peritoneal fat	—	—	*423*	401	377	*361*	349	332	316·5	302	—	283	270·5	—	Tail-off
			0·12	0·19	0·5	0·65	1·0	1·1	1·4	1·42		2·9	3·1		20

selectively over the region concerned. The remainder, known collectively as the "unsaponifiable matter," usually accounts for about 1–1·5 %[1] of the original oil, and (in an average cod-liver oil) has approximately the composition:

Cholesterol and higher alcohols	...	...	...	About	96 %
Vitamin A	...	...	...	...	,,	3–4 %
Ergosterol	...	...	...	...	(Order)	0·1 %
Vitamin D	...	...	...	...	,,	0·02–0·03 %

Cholesterol is practically diactinic; a 10 cm. layer of 4 % solution shows no selective absorption and only very little general absorption in the extreme ultra-violet. Similar considerations apply to the higher alcohols, as would be expected from their saturation; in fact neither cholesterol nor the higher alcohols can be detected spectroscopically in natural oils. The remaining three substances all show strong selective absorption.

Vitamin A, which is responsible for most of the absorption of liver oils in the near ultra-violet, has a characteristic broad band with a maximum at 328 $m\mu$. A portion of the observed intensity at 328 $m\mu$ is not due to vitamin A, as is shown by discrepancies between the vitamin A potency calculated from this value and from feeding tests and antimony trichloride blue values. However, since these discrepancies largely disappear when the unsaponifiable matter instead of the oil itself is used in the estimation [Coward *et al.*, 1931] it would seem that the interfering absorption is due to certain of the acid constituents, which are considered later. On the basis of the values: $E^{1\%}_{1\,cm.}$ 617 $m\mu$ 5000, 580 $m\mu$ 2600, and 328 $m\mu$ 1600, Carr-Price, 78,000, for apparently pure vitamin A [Carr and Jewell, 1933] an average good cod-liver oil contains about 0·04 % of vitamin A. The figure varies considerably from fish to fish and even more so from species to species, the range covered within our experience by mature fish

[1] This applies to the liver oil from most fishes, including the average cod, but certain fish-liver oils are so rich in one constituent (*e.g.* vitamin A in halibut; squalene in elasmobranch fishes) as to increase this figure considerably, up to 10 % or more in special cases.

being from 0·004 % in haddock-liver oil to about 10 % in a sample of halibut-liver oil representing the mixed oil from several livers. No typical value can therefore be given. We have considered these variations in a separate paper [Lovern *et al.*, 1933].

Ergosterol exhibits intense, highly selective absorption in the middle ultra-violet, with maxima at 293·5, 281·5, 270·6, 260, 250·3, 242 $m\mu$, $E^{1\%}_{1\,cm.}$ max. being of the order 290 (in alcohol). Thus with 0·001 % of ergosterol (a high figure for a fish oil) the absorption intensity (log I_0/I) in this region for a 1 cm. layer of a 1 % solution of the oil would be about 0·003, an obviously insignificant contribution to the U.V. absorption at 280 $m\mu$ usually observed, which ranges from 0·4 upwards in the typical fish-liver oils examined. This explains the observation of Morton *et al.* [1931] that the intensity of absorption in this region cannot be correlated with the sterol content.

Vitamin D (calciferol) in the pure state also absorbs strongly in the middle ultra-violet, having a single broad band, free from fine structure, with a maximum at 265 $m\mu$ ($E^{1\%}_{1\,cm.}$ 265 $m\mu$, 485). Askew *et al.* [1932] found a mean anti-rachitic value of 40,000 International Units per mg. of purified calciferol; typical cod-liver oils range from 25 to 200 units per g. Hence the vitamin D content of a cod-liver oil is, on this basis, of the order 0·00006–0·0005 %, giving for a solution of the oil an $E^{1\%}_{1\,cm.}$ value of 0·0003–0·0025, which obviously cannot influence the measured absorption curve of the oil. Even in puffer fish-liver oil, which it is stated [Bills, 1927] may be 15 times as rich in vitamin D as cod-liver oil, the effect would be inconsiderable.

Vitamin A, then, is the only non-acid material contributing appreciably to the ultra-violet absorption of most fish-liver oils. This, of course, is to be expected from the facts that it is both intrinsically more strongly absorbing than either ergosterol or vitamin D, and is also usually present in vastly greater proportions.

Upwards of a dozen different fatty acids combined as triglycerides are present in most fish-liver oils, a feature of which is their high content of unsaturated acids with 20–22 carbon atoms and four or more double bonds in the molecule. Though the proportions vary somewhat, saturated acids account on the average for 15–20 % of the total weight, mildly unsaturated (1–2 double bonds) about 70 %, and the highly unsaturated, including the so-called clupanodonic acids, 12–15 %. The precise composition of the more unsaturated fractions remains indeterminate by ordinary methods of analysis, in the sense that only the average degree of unsaturation is ascertained (expressed in terms of number of H atoms lacking as compared with the corresponding saturated acid) and not the individual unsaturation of the various members. Thus, to take a simple case, ($-2n$H) might imply one acid with n double bonds, or an equimolecular mixture of acids with $n-1$ and $n+1$ double bonds. In addition, the extent of conjugation of these double bonds is often a matter of uncertainty, particularly in the more unsaturated members. The latter, incidentally, have also been detected in several different kinds of algae, though in much lower proportions than in fish oils [Tsujimoto, 1925], and in the fat of birds which feed on fish, but not in birds which feed exclusively on insects or grain [Grün and Halden, 1929]. They are thus apparently connected in some way with aquatic life.

Turning now to spectral characteristics, oils which are known to consist mainly of saturated glycerides show either no selective absorption or at most only feeble indications of a group of three bands near 270 $m\mu$. Oleic acid, with one double bond, shows traces of the 270 $m\mu$ group which become fainter as the acid is purified. Linseed oil, which largely consists of the glycerides of

linoleic acid (order 50 %, F_2) and linolenic and *iso*linolenic acids (order 30–35 %, F_3) has an $E^{1\%}_{1\,cm.}$ 270 $m\mu$ value of 29, which is a mere fraction of that shown in this region by some fish-liver oil acids (*cf.* cod, monk) containing, as is usual, a rather smaller proportion of di- and tri-ethylenic acids than do the acids from linseed oil. It is therefore impossible to associate straight chain di- and tri-ethylenic acids with the highly selective absorption regularly observed at 270$m\mu$, and we are forced to the conclusion that the acid responsible for this group, if indeed it is a straight chain acid, must have more than three double bonds. This is not inconsistent with other considerations to be mentioned later, which further indicate that the remaining narrow bands exhibited nearer the visible region of the fish-liver oil acids must be associated with a still higher degree of unsaturation.

The C_{20}–C_{22} clupanodonic and higher unsaturated acids remain to be considered as possible causes of selective absorption. Here the mean unsaturation as determined by hydrogenation and iodine values is of the order F_{4-5}, but little appears to be definitely known concerning the extent of conjugation of these double bonds, without which little absorption of a selective nature is to be expected, in the absence of ketonic or other absorbing groups. According to Tsujimoto [1928] there is no conjugation of double bonds in a C_{20} acid which he terms clupanodonic, and which is known to possess a straight chain of carbon atoms from its conversion into behenic acid by hydrogenation; but the term "clupanodonic" is used somewhat loosely in the literature, sometimes being confined to the C_{20} and C_{22} acids with 4 or 5 double bonds, and sometimes including everything from C_{20-22} upwards[1]. The highly absorbing acids are evidently to be found somewhere among, or else are derived from, the polyethylenic higher acids. There are three possibilities: (i) that they are the "clupanodonic" *etc.* acids themselves; (ii) that they belong to a series of as yet unidentified compounds which, although perhaps very highly unsaturated, are present in such small quantities that they contribute little to the general unsaturation; (iii) that they are derived from the "clupanodonic" acids by isomerisation, cyclisation or other chemical change. In case (i), even if in some of the acids all the double bonds were conjugated, one would expect a series of relatively broad absorption bands, one from each acid, superposed to form an ill-defined summation curve, rather than the sharply defined narrow bands actually observed. Further, since the typical fish-liver oil of low free fatty acid content but rich in clupanodonic glycerides—using the term "clupanodonic" in its widest sense—is devoid of narrow bands, and since synthetic glycerides prepared from the highly absorbing total acids, including clupanodonic, *etc.*, exhibit the same fine structure as these acids, though at a slightly lower intensity [Gillam *et al.*, 1931] the interesting position arises that either the clupanodonic acids are not responsible for any of the observed fine structure, or the configuration of the acidic portion of the natural clupanodonic glycerides or esters differs from that of the clupanodonic acids themselves as prepared by thorough saponification of these glycerides [Morton *et al.*, 1931]. The same thing applies, of course, to any even more highly unsaturated acids which might be present.

Against case (ii) is the extraordinarily high extinction coefficients which must be postulated for the highly absorbing acids if they are present only in very small quantities.

Case (iii) provides the best working hypothesis, although it is not yet known with certainty whether the change in configuration (which appears to be irreversible) occurs during the saponification process or during the subsequent

[1] Inone and Sahashi [1932] give a $C_{22}F_5$ acid as containing an acetylenic linkage.

acidification. The evidence available points strongly to the former alternative and is supported by the recent work of Dann and Moore [1933], the results of these authors suggesting the existence of some connection between the length of time during which the alcoholic KOH solution is boiled (usually much longer than is strictly necessary for saponification) and the quantity of highly absorbing acids produced.

The possibility of an isomeric change in the acidic portion of certain glycerides giving rise to the narrow bands may account for a phenomenon which has been observed on more than one occasion. Sturgeon peritoneal fat containing an appreciable amount of vitamin A ($E^{1\%}_{1\,cm.}$ 602$m\mu$, 0·34; 568$m\mu$, 0·3) when first examined showed only two bands in the ultra-violet, one at 328$m\mu$ $E^{1\%}_{1\,cm.}$ 0·28, and one at 281$m\mu$, $E^{1\%}_{1\,cm.}$ 0·26, with no detectable fine structure. On re-examination some 4 months later bands were found at 460, 399 and 377 (inflexions), 325, 292, 282, 271$m\mu$, the 325–330$m\mu$ intensity remaining unchanged within experimental error ($E^{1\%}_{1\,cm.}$ 328$m\mu$, 0·27) while the rest of the bands ranged from $E^{1\%}_{1\,cm.}$ 0·0065 (460$m\mu$) to 0·54 (271$m\mu$). Similarly, a fresh solution of ling-liver oil (5 %) in heptane exhibited a broad band at 321$m\mu$ ($E^{1\%}_{1\,cm.}$ 0·5) and very faint traces of selective absorption at 398, 282 and 270$m\mu$. Two days later, unmistakable signs of selective absorption at 400, 377, 360, 348, 315, 282 and 270$m\mu$ were observable, together with a general increase in in-

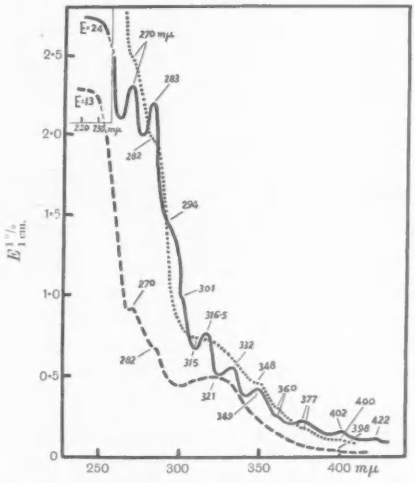

Fig. 1. Ling (*Molva vulgaris*). Solvent: heptane. $SbCl_3$ blue value of oil: $E^{1\%}_{1\,cm.}$ 603$m\mu$, 0·54; 578$m\mu$, 0·44.

— — — Liver oil, fresh solution.
..... Liver oil, same solution two days later.
——— Acids from saponification of liver oil.

tensity of absorption throughout (see Fig. 1). The new curve could in fact be accounted for by superposition of a low intensity ling-liver oil acid curve upon the original ling-liver oil curve.

Two interpretations are possible: either spontaneous hydrolysis takes place, or the fine structure-producing change can occur, without hydrolysis, in the

acidic portion of certain glycerides. It is probable that most of the fine structure observed in stale fish oils is due to one or both of these causes. Which cause predominates can be decided by washing with sodium carbonate and re-examining spectroscopically. That the change can occur within the glyceride molecule as a result of some process other than hydrolysis is proved by the fact that the narrow bands exhibited by a skate-liver oil were not eliminated as a result of washing with sodium carbonate. Further, the experience with ling-liver oil suggests that this non-hydrolytic change is favoured by dilution with a solvent, since the oil itself, which had only just started to show selective absorption apart from the vitamin A band, was by no means fresh.

It has not been possible to trace any parallelism between the vitamin A potency of an oil (as measured by the $SbCl_3$ colour test) and the intensity of absorption exhibited by the total acid fraction. Moreover, with increasing vitamin content the absence of such correlation becomes increasingly evident. It is therefore inferred that the fine structure acids are not decomposition products of vitamin A, a conclusion already arrived at by Morton *et al.* [1931] and supported by the appearance of narrow bands in the above-mentioned sturgeon peritoneal fat without sensible loss of intensity at $328 m\mu$. There is likewise no evidence to connect the acids in any way with vitamin D, and no direct connection has been traced between the acids and the sterol content [Morton *et al.*, 1931]. That the acids are directly associated with the synthesis of either vitamin A or D seems equally improbable for the same reasons, and also because a really fresh fish oil shows very little selective absorption apart from the $328 m\mu$ band, but the possibility of their being indirectly connected in some way with vitamin synthesis (*e.g.* as degradation or by-products of intermediate stages in the conversion of carotene) cannot at this stage be definitely excluded. Two significant facts stand out: first that liver oils are an incomparably better source of the highly absorbing acids than vegetable oils (*e.g.* olive, cotton-seed); second that, except when the diet contains minimum quantities of carotene, the conversion of carotene into vitamin A is a very wasteful process. It has not yet been explained what happens to the "wasted" carotene.

It will be recalled that the typical total acid absorption curve exhibits the following maxima: 230–$235 m\mu$ (always at a high intensity, sometimes a genuine maximum, more often a tail-off), (259), 270, 282, 288–295 (inflexion), 301·5, 316·5, 332, 348, 360 (inflexion), 377, 401 (422, 442, 460 inflexions or very weak bands), the figures in brackets being not always strictly reproducible. The question arises whether the whole of the fine structure is due to one acid or whether it is the result of superposition of a number of absorption curves, each arising from a different acid.

Evidence adduced from various sources all points to the conclusion that there are several absorbing entities. This evidence is summarised in Table II, in which the bands are segregated into discrete groups as far as the data warrant. Two different lines of attack have been followed, one based on an examination of the intensities of the various bands, to see whether there is any tendency for certain groups of bands to vary together in intensity, and the other based on frequency differences. The intensity method was applied to (i) the methyl esters of the more unsaturated cod-liver oil acids in which a partial separation had been effected by high vacuum distillation; details of this have already been published [Gillam *et al.*, 1931]; (ii) monk (angler-fish)-liver oil acids, which were partially separated (*a*) by adsorption on fibrous alumina, (*b*) by the Twitchell lead salt process, and (*c*) by distillation of the esters; (iii) the total acids in Table I. Incidentally, the separation of the monk acids by fibrous alumina

Table II. *Grouping of the maxima exhibited by the highly selectively absorbing acids.*

Former work*	Present work									
Fractionation of cod-liver oil acids (from intensity of maxima shown by different fractions)	Fractionation of monk-liver oil acids		Acids from several fish-liver and body oils. Theoretical considerations of							
			Intensity of maxima			Frequency differences				
234 $m\mu$	234 $m\mu$		230–5 $m\mu$			**230–5 $m\mu$**				
259	260		(259)			259				
270	270		270			**270**				
281	281		282			**282**				
				290 (inflexion)		295	288			
(301)	301			301·5			**301·5**			
(316)	316·5			316·5			**316·5**	317·5		
	(331)	(331)			332		333	**332**		
		(349)			348			**348**		
		—			360 (infl.)			365·5	357	
		375			377				**377**	
		394			401				**401**	
									427	
			Δ cm.$^{-1}$ = 1580,		1570,		1380,		1590	

* Gillam *et al.* [1931].

gave the highest all round values yet recorded for the intensities of the narrow bands $E^{1\%}_{1\,\text{cm.}}$ 269 $m\mu$, 380; 234 $m\mu$, 440.

When a broad absorption band can be resolved into a number of narrow bands, the separation (in wave-numbers) of the components is often nearly constant, the Δ cm.$^{-1}$ value corresponding with a vibrational frequency. The provisional grouping of the maxima shown by our acids can be tested by applying this criterion. Thus if the 270 and 282 $m\mu$ bands are physically related (Δ cm.$^{-1}$, 1580), bands would also be expected at 259 and 295 $m\mu$; and similarly for the other groups in Table II. The figures in heavy type represent the dominant regularly observed bands, while the others are the calculated wave-lengths of the fainter bands which one would expect to be associated with the main bands. This particular segregation into groups agrees very well with the main facts and provides a plausible explanation of a number of otherwise apparently anomalous features.

(i) The 259 $m\mu$ band is not always observed. This is to be expected if it is both intrinsically faint and occurs on a steep part of the curve due to the 230–5 $m\mu$ substance, by which it is likely to be masked or at most reduced to a faint inflexion. It should perhaps be explained that a weak band superposed on a steep absorption curve due to another substance appears as an inflexion on that curve.

(ii) A long inflexion, varying in position, is often found about 290 $m\mu$. This can be attributed to the joint effects of the 288 and 295 $m\mu$ faint bands, the middle of the inflexion being nearer to 288 or 295 $m\mu$ according to which substance predominates.

(iii) The overlapping of the two middle groups similarly explains variations of 0·5–1·0 $m\mu$ in the position of the 316·5 and 332 $m\mu$ bands according to the source of the oil from which the total acids were derived.

(iv) The 360 $m\mu$ inflexion is apparently due to the superposition of the two faint 365·5 and 357 $m\mu$ bands, and, forming a link between two sets of bands, accounts for the tendency to group them together as one when intensity data only are considered.

(v) By calculation, a band should appear at 427 $m\mu$. In practice, one is observed, either as an inflexion or as a weak band, near 423 $m\mu$. This may be due to interference by a trace of something analogous to the monk pigment [Lovern and Morton, 1931] one of the absorption bands of which lies at 422 $m\mu$. It should be remembered that the intensity of this part of the "total acid" absorption curve is very low and the curve correspondingly more susceptible to such influences.

There are thus probably at least five highly absorbing entities contributing to the complex absorption curve of the total acids. At this stage we can only speculate as to their inter-relationship and structure, but there are certain general conclusions which can be regarded as not improbable. In common with the other acid constituents, they all appear to be monobasic. This is suggested by the behaviour of the total unsaturated acids on esterification with methyl alcohol and sulphuric acid. In general, monobasic acids esterify readily and almost completely (95–98 %), while dibasic acids show a tendency to stop short at one carboxyl group (*i.e.* 50 %). The acids from fish-liver oils esterify with the same ease and to about the same extent as normal monobasic acids, showing that in the main, at least, the acids are monobasic. Further, the small "unesterified" portion, in which any partially esterified dibasic acids would collect if present, shows no increase in unsaturation or absorption, thus indicating that the fine structure acids themselves also are monobasic.

Concerning the absolute quantities of the highly absorbing acids present, little can be said at the moment. The percentage of so-called highly unsaturated acids, C_{20-22}, with four or more ethenoid linkages, shows no correspondence with the observed range of $E^{1\%}_{1\,\text{cm.}}$ values. Thus the 270 $m\mu$ group of bands has usually an $E^{1\%}_{1\,\text{cm.}}$ value of 2 or 3 in the acids from the liver oils of most of the species studied, and yet in certain cod-liver oil acids, with about a normal percentage of C_{20-22}, F_{4-5} upwards, the E value was 250. Since the cod-liver oil acids evidently contained 100 times the normal amount of these fine structure acids, and this was not reflected in a measurable increase in the general unsaturation of the polyethylenic fraction, the inference is either that the fine structure acids are present only in minute quantities, or that the change from relatively diactinic unsaturated acids to highly absorbing unsaturated acids leaves the iodine value practically unchanged.

The monk-liver oil acids, which were examined in considerable detail, support these general conclusions. Their approximate composition is as follows:

	%
Saturated acids:	
Myristic	4·9
Palmitic...	9·6
Stearic	1·3
Unsaturated acids:	
Myristoleic	0·4 (−2·0 H)
Palmitoleic	12·1 (−2·0 H)
C_{18} group	30·9 (−3·3 H)
C_{20} group	24·9 (−5·9 H)
C_{22} group	15·9 (−8·6 H)

They thus fall in line with the fatty acids from the majority of marine fish-liver oils as regards general composition, but are unusually interesting in that they are associated with a red pigment in the liver oil [Lovern and Morton, 1931] and are from 10 to 30 times as highly absorbing in the ultra-violet as the usual

total acids from most fish-liver oils. Like the cod-liver acids, their high absorption is not reflected in the measured unsaturation, and they give no colour reaction with antimony trichloride. Distillation of the methyl esters under 0·1 mm. pressure results in a very imperfect separation of the highly absorbing constituents, but there is a marked tendency for them to accumulate in the high-boiling fractions and in the residues, a fact which suggests a high molecular weight, probably of the order 350.

Table III summarises spectroscopic data on certain of the simpler unsaturated compounds with conjugated ethenoid linkages, the data on the acids being due to Kuhn (private communication) and on the hydrocarbons to

Table III.

		λ max. $m\mu$	log ε max.
Acids			
Crotonic	$CH_3CH=CHCOOH$	204	4·085
Sorbic	$CH_3(CH=CH)_2COOH$	254	4·398
Octatrienecarboxylic	$CH_3(CH=CH)_3COOH$	294	4·556
Decatetraenecarboxylic	$CH_3(CH=CH)_4COOH$	329	4·690
Hydrocarbons			
αα′-Diphenylbutadiene	$C_6H_5(CH=CH)_2C_6H_5$	325	3·65
αα′-Diphenylhexatriene	$C_6H_5(CH=CH)_3C_6H_5$	371	3·8
		355	3·83
		333	3·7
αα′-Diphenyloctatetraene	$C_6H_5(CH=CH)_4C_6H_5$	396	3·87
		372	3·86
		357	3·80
αα′-Diphenyldodecahexaene	$C_6H_5(CH=CH)_6C_6H_5$	441	3·73
		418	3·73
		380	3·7
		317	3·2

Rădulescu and Bărbulescu [1931]. Two general tendencies are observable: (i) an increase in the number of conjugated double bonds causes a shift of the ultra-violet absorption maxima, or group of maxima, towards the visible; (ii) this shift is usually accompanied by an increase in absorption intensity. The groups of bands exhibited by the series of hydrocarbons due to the combined influence of ring systems and conjugated double bonds show a qualitative overlapping similar to that already mentioned in connection with the "fine structure" acids, indicating the possibility of a similar series relationship between the acids, the quantity present decreasing as the extent of conjugation increases. This would account for the general shape of the absorption curve of the total acids, which falls as the visible is approached, instead of rising as would be expected if equal quantities were present.

Table IV records the absorption maxima of a number of unsaturated substances connected with the whole question of fat-soluble vitamins. It will be observed that the bands in the visible associated with the highly unsaturated acids are reminiscent of those shown by carotenoids and polyene acids, whilst the ultra-violet bands resemble those shown by polycyclic, partially hydrogenated substances, the resemblance being closest for "dihydro" carotene (prepared by the action of aluminium amalgam on carotene) and the cyclised product derived from vitamin A and hydrochloric acid [Edisbury *et al.*, 1932]. The highly unsaturated acids probably contain appreciable quantities of substances with tricyclic skeletons, a view which receives some support when the absorption spectra of anthracene and phenanthrene derivatives are considered.

Table IV.

Total acids	—	460	445	422	401	—	377	360	348	332 mμ
Vitamin A+HCl	—	—	—	420	—	392	—	369	350	333
"Dihydrocarotene"	—	461	426–45	—	—	392	—	369	350	332
Carotene	492	461	436	—	—	—	—	—	348	—
Bezssonoff's extract*	476	—	448	426	408	386	—	—	349	330
α-Crocetin	—	—	448	420·5	399	—	—	—	—	—
Dihydrocrocetin	—	—	—	—	—	—	382	361·5	344	—
Monk (angler-fish) pigment	480	—	450	422	—	—	—	—	—	—
Ergosterol	—	—	—	—	—	—	—	—	—	—
Dehydro-ergosterol	—	—	—	—	—	—	—	—	342	325·5
Ergosterol D	—	—	—	—	—	—	—	—	—	—

(*continued*)

Total acids	316·5	301·5	290	282	270	259	—	—	230–5 mμ
Vitamin A+HCl	313–20	—	292	280	272	261	254	—	—
"Dihydrocarotene"	316	301	—	—	—	—	—	—	—
Carotene	—	—	—	280	—	—	—	—	—
Bezssonoff's extract*	—	300	—	283	—	—	—	—	—
α-Crocetin	310	—	—	—	—	—	249·5	—	—
Dihydrocrocetin	320–6	—	—	—	274·5	—	—	—	—
Monk (angler-fish) pigment	—	—	—	—	—	—	—	—	—
Ergosterol	—	—	293	281·5	270	260	250·3	242	—
Dehydro-ergosterol	311	—	296	—	—	—	—	—	—
Ergosterol D	—	—	—	—	—	—	252	243	235

* A residue, kindly furnished by Prof. Bezssonoff, from carotene extracts.

The position now reached may be stated briefly.

1. The well-defined narrow absorption bands owe their origin to unsaturation in the absorbing molecules.

2. The highly absorbing acids are not present in the original oils as the corresponding glycerides.

3. The hypothesis that the absorbing acids are straight chain compounds with conjugated double bonds is not in harmony with the evidence.

4. A change, unsaturated acids (low absorption) → unsaturated acids (high and selective absorption) occurs during saponification.

5. Cyclisation is the most plausible explanation of this change.

Further work is proceeding on these lines.

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CXCVII. VARIATIONS IN VITAMIN A CONTENT OF FISH-LIVER OILS, WITH PARTICULAR REFERENCE TO SEASONAL FLUCTUATIONS IN THE POTENCY OF HALIBUT-LIVER OIL.

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The object of the present work was initially to gather data concerning the vitamin A potency of the liver oils obtained from various species of fish. It was soon found that the vitamin A content of halibut-liver oil was remarkably high in comparison with oils from other species, and the scope of the investigation [*cf.* Lovern, 1932] was widened to embrace the elucidation of the factors capable of influencing the vitamin A content.

Collection of material. Livers were obtained mainly from commercial line-fishing vessels operating from Aberdeen and fishing in waters near Iceland, the Faroes and N.W. Scotland. The average duration of a trip was 2–3 weeks, and in each case the livers from all catches were mixed and stored at 0° in tins holding about 22 lb. The livers were received in varying states of preservation; some were very fresh and firm, whilst in other cases the contents of a tin had become a semi-liquid mass from which individual livers could not be separated. Each batch of livers was minced, mixed well, dried with anhydrous sodium sulphate and extracted with ether, and a dark sticky oil was finally obtained.

The halibut livers varied in weight from 4 oz. to 7 lb., but the great majority were between the limits of 12–20 oz., so that 1 lb. represents the approximate average weight of the halibut livers obtained by us in Aberdeen.

Towards the end of 1932 it became impossible to purchase halibut livers directly, as the production had been acquired in advance for the commercial extraction of the liver oil, but through the courtesy of Messrs Isaac Spencer and Co., Ltd., and Messrs Allen and Hanburys, Ltd., quantities of liver (10–14 lb. at a time) were purchased periodically. During the earlier period when supplies were obtained direct from the trawlers, arrangements were made for the skippers to note the position of the fishing ground, the nature of the stomach contents and the size of the halibut represented by the catch. As a rule, the livers in a batch were of assorted sizes, but occasionally fairly homogeneous catches of large or small halibut were obtained and the livers were classified as "mainly large" or "mainly small." The position of fishing grounds was also ascertained for the livers purchased from commercial houses during the later period. Through the courtesy of Messrs Parke, Davis and Co., we were also able to examine representative samples of commercial halibut-liver oil prepared from the livers of fish caught in Pacific waters and in the Davis Straits (*vide infra*). Much of

the data obtained is unsuitable for a detailed report, but the following main conclusions may be quoted.

(i) No very precise correlation between vitamin A potency and the position of the fishing ground can be established, although there are indications that higher maximum values are obtained in the liver oils from halibut caught in more northerly waters, probably reflecting the tendency of the larger fish to migrate northwards.

(ii) No significance can be attached to the stomach contents or the intensity of feeding [see Lovern and Sharp, 1933].

(iii) There is considerable evidence that the vitamin A potency of halibut-liver oils (in common with those of several other species) increases with increasing size of liver, and therefore age of fish.

(iv) Well marked seasonal variation was shown.

Vitamin A tests. Rough Carr-Price determinations were carried out at Aberdeen on the fresh oils, followed by spectrometric assay at Liverpool, using the Hilger-Nutting spectrophotometer for the antimony trichloride colour test, and Hilger E 3 spectrographs for the direct determination of vitamin A content on the basis of intensity of absorption in the ultra-violet shown by alcoholic solutions of liver oil.

Of all the constituents of fish-liver oils, vitamin A is by far the most variable in concentration. This is true not only in comparing oils from different species, but also as reflecting the differences between specimens of oil obtained from individuals of the same species. Our experience makes us unable to attach any precise significance to the idea of a fish-liver oil "typical" as regards vitamin A potency.

Variations in vitamin A content from species to species, arising possibly from different requirements and feeding habits, are perhaps only to be expected, but their magnitude is nevertheless surprising (see Table I). The percentages are calculated on the basis of the richest vitamin A distillates [*cf.* Carr and Jewell, 1933]:

$$E^{1\%}_{1\text{cm.}} \begin{cases} 617 m\mu,\ 5000 \\ 580 m\mu,\ 2600 \end{cases} \text{antimony trichloride colour test.}$$

328 $m\mu$, 1600 direct ultra-violet absorption test.

78,000 Carr-Price units.

The samples of oil from some species represent only a relatively small number of livers, so that considerable variations from our figures may be possible in later tests. Nevertheless, experience up to the present points to the conclusion that the liver oils of haddock, whiting, skate of small or medium size, codling and immature or small fish generally are markedly inferior in vitamin A potency to average cod-liver oil; the oils from pollack, saithe, hake and ling (probably also torsk) are usually similar in potency to cod-liver oil and subject to roughly the same variations; salmon, turbot, sturgeon and halibut yield liver oils which are vastly richer in vitamin A than cod-liver oil. The richer the source the more widely does the potency appear to vary as between sample and sample.

According to the data of Bills [1927] the over-all range of antirachitic activity of liver oils is some 500:1, the majority of oils falling within a range of 30:1. Vitamin A has a wider range, at least 2500:1, and, so far as we can ascertain, no parallelism between vitamin A and vitamin D potencies can be substantiated. At present, we have no views to submit regarding the significance of a 2500:1 range in vitamin A content; we are more concerned to ascertain the facts relating to variations (within a given species) in batches of oil, each representing

Table I. *Vitamin A content of fish-liver oils.*

Liver oil	Estimated percentage of vitamin A in the samples examined
Catfish (*Anarrhichas lupus*)	0·06
Cod (*Gadus callarias*) (mean of 43 samples)	0·04
Codling (*G. callarias*) (smaller specimens)	0·004
Codling (*G. callarias*) (larger specimens)	0·008
Haddock (*G. aeglefinus*)	0·004
Pollack, lythe (*G. pollachius*)	0·04
Saithe, coalfish (*G. virens*)	0·09
Whiting (*G. merlangus*)	0·005
Conger eel (*Conger vulgaris*)	0·25
Dab (*Pleuronectes limanda*)	0·015
Lemon sole (*P. microcephalus*)	0·07
Plaice (*P. platessa*)	0·1
Hake (*Merluccius vulgaris*)	0·04
Halibut (*Hippoglossus vulgaris*) (highest recorded, 1932)	10·0
(*H. vulgaris*) (lowest recorded, 1932)	0·17
Ling (*Molva vulgaris*)	0·015
Monk (angler-fish) (*Lophius piscatorius*)	0·02
Newfoundland flat-fish (*Platysomatichthys hippoglossoides*)	0·04
Pike (*Esox luscius*)	0·08
Rat-fish (*Chimera mirabilis*)	0·008
Salmon (*Salmo salar*)	0·3
"Blue" skate (*Raia batis*)	0·007
Thornback skate (*R. clavata*) (small specimens)	0·01
(*R. clavata*) (large specimens)	0·05
Sturgeon (*Acipenser attilus*)	0·8
Torsk, tusk (*Brosmius brosme*)	0·08
Turbot (*Psetta* (*Rhombus*) *maximus*)	0·3

too many livers to have any meaning as regards individual fishes but large enough, it is hoped, to be significant as regards the major causes of fluctuations and variations in potency.

Two distinct kinds of variation are discernible, progressive and seasonal, the seasonal being superimposed on the progressive variation; and without excluding the possibility of less obvious factors, the following variables appear to be important:

(i) Size (or age) of fish.
(ii) Sexual condition.
(iii) Diet.

The progressive aspect of the vitamin A variability seems to depend on (i), whilst (ii) and (iii) appear largely to account for the seasonal changes.

Within a given species we have found that large livers yield as a general rule a more potent oil than small livers, and our qualitative observations are supported by the recent more quantitative work of MacPherson [1933], who has found a linear relationship between age of fish and the potency of cod- and American plaice- (*Hippoglossoides platessoides* Fabricius) liver oils. As the livers of the larger members of a species usually represent a greater proportion of the total body weight than is the case with smaller specimens, and as, further, the larger livers generally yield a higher percentage of oil, the total vitamin A reserve of the fish increases rapidly with size. This probably results from a purely mechanical storage of the excess of vitamin (ingested as such or synthesised *in vivo* from carotene) over the requirements of the fish. Be the explanation what it may, the age factor is of considerable importance. Thus in the cod, the ratio between vitamin A potencies for mature fish and codling is about 10:1,

corresponding to about 20 or 30:1 in the actual weight of vitamin A per unit weight of fish. Qualitatively confirmatory evidence is available for skate and halibut.

It has long been recognised that during the spawning period changes occur which may profoundly affect the vitamin content of the liver oil. The cod, for example, eats little or no food at such times, and the drain on fat-reserves incidental to fasting is increased by the utilisation of large quantities of liver oil in the development of the genital products [*cf.* Hjort, 1914]. A certain amount of vitamin A is transferred with this oil to the gonads [Zilva *et al.*, 1924], and the total vitamin reserves are to that extent depleted; but the residual liver oil is so reduced in quantity that the concentration of vitamin A in the oil is higher than when the fish is feeding normally. It appears, in fact, that at the spawning period both vitamin A and D potencies vary approximately inversely as the oil content of the liver [Drummond and Hilditch, 1930]. This utilisation of liver oil at spawning would of itself give rise to a peak in the potency curve immediately after spawning. Resumption of feeding results in gradual dilution of the liver oil as fat is again stored, the drop in potency being mitigated by replenishment of vitamin A reserves. The average percentage of oil in fish-livers varies from species to species (*e.g.* halibut 18–20 % (average); monk or angler fish, 40 %; certain gadoid fishes, up to 70 %) as also does the range over which the percentage of oil varies for the pre- and post-spawning periods, but the range rarely exceeds 2 or 3 to 1 for the spawning effect. The males of the various species are much less affected than the females.

Diet may next be considered as a contributory cause of fluctuations in potency. In a sense, diet is fundamental, since there is no evidence of direct synthesis of vitamin A by fishes as appears to be possible with vitamin D in some species [*cf.* Bills, 1927], and in any event the richer the diet, the greater are the vitamin reserves likely to be. Drummond *et al.* [1922], Jameson *et al.* [1922] and Hjort [1922] traced the fish-liver vitamin A through a series of intermediate stages to the diatoms (unicellular vegetable organisms in the plankton), which are able like land plants to produce carotene as a result of photosynthesis. There is as yet no evidence that diatoms or the minute planktonic animal organisms (copepods, larval forms of various marine creatures, *etc.*) which subsist on diatoms, contain vitamin A as such, or vitamin D. The published biological assays of the growth-promoting effects of planktonic organisms exhibit curiously good agreement in some cases, and very bad agreement in others, so that seasonal variations may perhaps be suspected here also. In any case, the plankton exhibits very well-marked seasonal fluctuations in abundance, an effect due primarily in the case of diatoms to seasonal changes in the intensity of sunlight and less obviously to fluctuations in silica, nitrogenous matter and carbon dioxide in the sea water. The curves in Fig. 1 show the mean annual changes in the abundance of planktonic flora and fauna generally in Port Erin Bay for the period 1907–1920, based on data given by Johnstone *et al.* [1924], and are fairly representative for the whole of the northern hemisphere, with the reservation that in 1932 the spring maximum of the diatoms may have been a few weeks earlier than usual (in Plymouth Sound this occurred in March). It would appear from the curves that the greater part (probably 80 %) of the total amount of carotene annually produced by the diatoms is synthesised in the spring or early summer, with a comparative scarcity, so far as initial synthesis is concerned, during the rest of the year. A time-lag of uncertain duration must exist before this carotene reappears in the form of vitamin A in the liver oils of large fishes, owing to the number of links in the

food-chain. The spring maximum must first of all affect the copepods and larvae living on diatoms; next, immature fish and small mature fish (*e.g.* herring, caplin), certain molluscs and other organisms which subsist on copepods; thence, either directly or through one or two further stages, the larger fish. The effect is, moreover, liable to be wholly or partly obscured by other factors such as the presence of large reserves of liver oil and changes in oil content due to spawning. Indeed, the last factor gives rise to the only seasonal fluctuation which can be discerned at all in the majority of species. The halibut, apparently, is an exception.

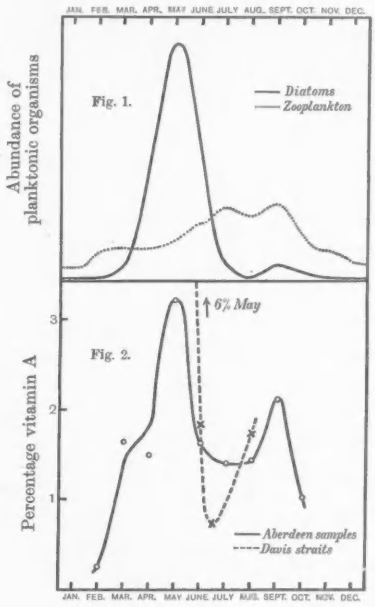

Fig. 1. Mean annual plankton variations, 1907–20, Port Erin Bay; adapted from Johnstone *et al.* [1924].

The diatom curve is roughly quantitative. The zooplankton curve, which is more diagrammatic, reflects the July and September maxima due to copepods, the main curve being modified by larvae (early spring and November) and protozoa (June–July).

Fig. 2. Seasonal variations in vitamin A content of halibut-liver oils (1932).

Insufficient samples were obtained during March to justify drawing the curve with a further subsidiary maximum in the early spring.

Tables II and III summarise data which have been obtained during 1932 on halibut-liver oils. Some comment must be made on the statistical significance of the results in the last columns of Tables II and III, from which the curves in Fig. 2 were drawn. The halibut livers from Aberdeen are representative samples of catches made during the greater part of the year, and not less than 1500 livers were worked up on a laboratory scale over the whole period. The material from the Davis Straits was obtained from some 100,000 livers (4500

Table II. *Halibut-liver oils (samples from Torry Research Station).*

Mean date of catch	Approx. total wt. of livers per batch in lbs.	Carr-Price B.U.	$E^{1\%}_{1\,cm.}$ 617 mμ	$E^{1\%}_{1\,cm.}$ 580 mμ	$E^{1\%}_{1\,cm.}$ 328 mμ	Estimated % vitamin A	Aberdeen samples % vitamin A for month (weighted mean)
12. ii. 32	30	—	10·8	7·7	5·5	0·30	
15. ii. 32	30	—	12·1	8·7	6·0	0·34	0·26
22. ii. 32	30	60	7·5	6·0	4·25	0·23	
23. ii. 32	30	60	6·2	4·8	3·5	0·17	
7. iii. 32	30	—	70	47	30	1·9	
10. iii. 32	30	1600	139	80	42	2·8	1·65
16. iii. 32	30	71	10	6·5	5·0	0·25	
5. iv. 32	30	66	8·2	5·5	—	0·2	
18. iv. 32	30	1030	—	—	—	*ca.*2	
18. iv. 32	30	1100	—	—	—	*ca.*2	
24. iv. 32	33	820	67	44	26	1·6	1·5
27. iv. 32	16	540	42	29	19	1·1	
28. iv. 32	33	1900	132	89	50	3·1	
30. iv. 32	44	210	17·3	14·6	—	0·5	
3. v. 32	44	205	25	14·5	80	0·5	
16. v. 32	30	2770	176	98	57·5	3·6	
17. v. 32	33	3030	241	194	80	5·0	3·23
18. v. 32	22	7630	430	260	164	10·0	
20. v. 32	30	780	72·7	44·5	24	1·5	
21. v. 32	30	865	90	49	27·5	1·7	
4. vi. 32	30	285	18	14	8·5	0·53	
10. vi. 32	30	890	113	75	39·3	2·5	1·63
12. vi. 32	30	625	45	29·5	15·4	1·0	
14. vi. 32	30	1240	121	74	36·5	2·5	
1. vii. 32	30	290	20·8	12·4	6·8	0·42	
3. vii. 32	30	1170	124	73	35	2·45	
16. vii. 32	30	1800	175	101	47·5	3·4	1·41
18. vii. 32	77	775	85	52	23	1·7	
30. vii. 32	200	500	52	33·5	15	1·0	
3. viii. 32	—	270	27	17	8·6	0·55	
3. viii. 32	—	1365	148	90	—	3·1	
3. viii. 32	33	480	58	35	19	1·2	
9. viii. 32	14	125	16·4	9·7	6·4	0·38	
9. viii. 32	14	1870	188	108	58	3·75	
10. viii. 32	—	1340	165	98	45	3·3	
10. viii. 32	—	385	34	22	11	0·75	1·44
12. viii. 32	44	210	17·5	12·5	8·75	0·53	
14. viii. 32	—	3000?	134	81	42	2·8	
15. viii. 32	132	950	77	44·5	24·4	1·6	
19. viii. 32	14	240	20·2	14	8·7	0·5	
24. viii. 32	14	525	58	34	19·2	1·25	
30. viii. 32	110	355	37	20	12·3	0·8	
7. ix. 32	14	1080	83	50	25·5	1·7	
9. ix. 32	30	1510	159	87	50	3·2	
9. ix. 32	14	685	78	44	24	1·6	
12. ix. 32	14	640	61·5	34	21·7	1·3	
13. ix. 32	14	1640	126	72	41·2	2·7	
22. ix. 32	14	610	54	34	16·6	1·1	
24. ix. 32	14?	165	13	8·5	5·5	0·33	2·12
30. ix. 32	44	1750	166	94·5	48·7	3·2	
30. ix. 32	166	1000	98	55	30	2·0	
30. ix. 32	—	1740?	102	60	32	2·2	
30. ix. 32	—	975	71	44·5	24	1·55	
30. ix. 32	—	895	59	34·6	19	1·25	
30. ix. 32	—	800	52·4	30·4	17·1	1·1	
30. ix. 32	—	1470	86	52	29·5	1·8	
7. x. 32	14	385	34	24	15·3	0·95	
13. x. 32	14	420	40	24	14·5	0·90	1·02
21. x. 32	14	160	13·9	8·8	5·4	0·32	
26. x. 32	—	1530	90	50	31	1·9	

Table III. *Halibut-liver oils from the Davis Straits.*

Fish caught (1932)	Carr-Price B.U.	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	Vita-min A %	$E^{1\%}_{1\,cm.}$ 580 $m\mu$	Vita-min A %	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	Vita-min A %	Mean % vita-min A
May	4700	300	6·0	170	6·54	90	5·62	6
June	1400	90	1·80	49	1·88	28·7	1·80	1·83
June–July	630	37	0·74	19	0·73	12	0·75	0·74
August	1150	85·4	1·71	47	1·81	27·5	1·72	1·74

tons of fish) and the livers worked up on a technical scale. The results of the small scale and large scale treatments are consistent. The monthly average vitamin A potencies of the samples obtained from Aberdeen exhibit a very sharp maximum in May and a subsidiary maximum in September, and the curve for the Davis Straits samples is qualitatively similar over the period for which the data are available.

Discussion.

The interpretation of Fig. 2 is a matter of some difficulty. The observed fluctuations in monthly average potency cannot be attributed entirely to variations in oil content due to spawning. In the first place, the range of potency covered by the Davis Straits samples is about 8:1, whereas all the oils examined have represented between 12 and 35 % of the liver weight (*i.e.* a range of 3:1), the great majority lying between 15 and 25 %. Secondly, the seasonal fluctuation in potency is just as marked and shows the same tendency to the same two maxima whether spawning occurs in February–May (N.W. Scotland) or June–August (northern waters). For this reason all the data for the Aberdeen oils have been combined in one curve, whether the fish were caught off Iceland, the Faroes, or Scotland, and the "statistical" halibut represented by this curve may be regarded as spawning over the whole period from February to August.

Unless there is some more obscure process operating on or within the halibut, the only remaining likely cause of seasonal fluctuations in potency is the variation in available carotene, and it seems significant that the only measure we have of this factor, namely the abundance of diatoms, should show, qualitatively at all events, such a strikingly similar curve (Fig. 1). We hesitate to stress the parallelism unduly without further confirmatory evidence, and a completely convincing correlation is naturally very difficult to achieve since the halibut does not subsist directly on diatoms. Nevertheless, it seems to us highly probable that when carotene (in the plankton) is abundant, vitamin A reserves are being replenished by all fish, and that when these supplies are small the reserves are being drawn upon. The effect on liver oil potency will not be very marked in most fish, but the halibut appears to reflect this ebb and flow in so marked a way as to obscure even the spawning factor. The fact that the vegetable plankton is richer in colder waters and that the halibut-liver oils from the Davis Straits reached in May an average potency nearly twice as high as the Aberdeen samples, is not inconsistent with this view, though the larger size of fish may also account for the phenomenon. If the latter view is correct, it would appear that the larger the fish, the greater the fluctuation, as is shown by the low minimum reached by the Davis Straits curve.

The curves further suggest an enormous consumption of reserve vitamin A by the halibut during the summer and late autumn, and the possibility cannot at present be excluded, as an alternative explanation, that the potency fluctuation may arise from a rhythmic transference of vitamin A between the liver

and some other part of the body, influenced by some unknown factor which varies seasonally in the same way as the diatoms. All this, of course, is pure speculation, but whatever their origin the fluctuations themselves seem real enough. It remains to be seen whether the halibut-liver oil curves published here are reproducible from year to year, subject, of course, to minor variations in time and intensity. We have, however, noticed the same general tendency for two successive years, and there is no reason to believe that they were exceptional.

The over-all range in variation of vitamin A in halibut-liver oils is seen from Table II to be certainly not less than 60:1 (10 to 0·17 %); more probably, since the samples each consisted of mixed oils from a number of livers, nearer 100:1, a much larger range than has been observed in any other species. Seasonal fluctuations account for only a small proportion of this range, and variations in the age of the fish caught (which, we are informed, would be from about 12 to 40 years) may here again be the dominant factor, as it appears to be in the case of cod [MacPherson, 1932; 1933].

We are, in short, led to infer that the oil obtained from any given halibut liver increases in mean annual potency as the fish grows older, and in addition fluctuates from season to season over a gradually increasing range. Further, the extraordinarily high proportion of vitamin A in some of the samples, together with these wide fluctuations in concentration, suggests that, at least in the halibut metabolism, vitamin A may play an additional rôle to that usually associated with the term "vitamin."

Summary.

1. The vitamin A content of fish-liver oils covers a range of at least 2500 to 1. No parallelism can be traced between the vitamin A and vitamin D potencies.

2. In certain species, the vitamin A content of the liver oil has been found to increase with the age and/or size of the fish, the total vitamin A reserve increasing more rapidly than the oil potency.

3. Attention is drawn to the fact that the greater part of the carotene annually produced by the diatoms, and thus available for conversion into vitamin A by marine animal life, is initially synthesised during a comparatively short period in the spring or early summer.

4. Halibut-liver oil is by far the richest known natural source of vitamin A available in quantity, but it has been found to vary in potency over a wider range than any other source. Oils containing from 0·17 to 10 % of vitamin A have been examined and these apparently do not represent extreme limits. No correlation has emerged between the immediate diet of the halibut and the oil potency [Lovern and Sharp, 1933].

5. Halibut-liver oils have been found to exhibit well-marked seasonal fluctuations in vitamin A concentration which cannot be attributed to changes in the oil content of the liver occasioned by spawning. The explanations offered are necessarily tentative, but the fluctuations themselves are quite definite.

6. The best oils from the standpoint of vitamin A content are most likely to be obtained from large halibut caught in northern waters in the late spring or early summer, and in the autumn. Very rich oils at other times of the year are exceptional. (*Mutatis mutandis* this may also apply to the southern hemisphere.)

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CXCVIII. THE DIET OF THE HALIBUT AND INTENSITY OF FEEDING, IN RELATION TO THE VITAMIN A POTENCY OF THE LIVER OIL.

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(*Received July 14th, 1933.*)

In an endeavour to elucidate the factors responsible for the observed wide variations in the vitamin A potency of halibut-liver oil [Lovern *et al.*, 1933], examinations were made of the stomach contents of a number of fish. The method adopted was to furnish the skippers of the vessels supplying the livers with a typewritten form, on which, amongst other information, they wrote down the nature of the stomach contents (as far as ascertainable) of all their catch of halibut. Any material which they could not readily identify was placed in bottles of formalin, supplied for the purpose, and brought back for identification at the Torry Research Station.

It was realised from the outset that food found in the stomachs only gave evidence of the nature of the last meal, and was no clue to the type of food on which the fish had been feeding for some time. However, it was hoped that some light might be thrown on the source of the unusually large supplies of vitamin A present in the liver of the halibut—even in the poorest specimens. The results, however, were entirely negative. In many cases the stomachs were empty, but the actual food found was of so varied a nature as to suggest that the halibut ate anything they could get. The commonest species found in the stomachs were torsk, dogfish, catfish, small dabs, and crustaceans, with many other specimens, such as megrims, ling, *Sebastes norvegicus, etc.* In some cases remains of herring were found, but it could not be decided whether these had come from the bait or not.

It was evident from the vitamin A potencies of the liver oils (as measured by the antimony trichloride reaction) that no relationship could be adduced between high potency and any particular diet. None of the species found in the stomachs is an unusually rich source of vitamin A, although the one sample of torsk-liver oil which was examined was superior to most cod-liver oils. It may be worth noting that torsk was found in the stomachs more frequently than any other species.

As the diet seemed to be of so general a nature, with no outstandingly rich supply of vitamin A, it was decided to try to get some measure of the intensity of feeding. For this purpose, the glycogen content of the livers was chosen as a criterion. Admittedly it has obvious drawbacks. It is not known, for instance, to what extent intensive feeding, on a diet mainly of proteins and some fat, would affect the liver glycogen content, nor is it known to what extent, or how quickly, struggling of the fish on the line would deplete the liver reserves of

glycogen. In any event, wide variations in glycogen content were experienced, so large, in fact, that it seems the most feasible explanation to ascribe them to dietary causes. Even so, it is extremely doubtful if the values have reference to anything more remote than the last meal of the fish, but no other means of investigating intensity presented itself.

From fish brought aboard a great-line vessel fishing the Faroes grounds the livers were immediately cut out and weighed, a sample of each being preserved in 60 % KOH for estimation of glycogen on return of the vessel to port. The remainder of the livers, used for oil and vitamin A analyses, was stored in ice in two batches; A, all livers under $\frac{3}{4}$ lb. in weight, B, all livers over $1\frac{1}{2}$ lbs. in weight. The data obtained from the first two experiments are given in Table I. Vitamin A potencies are expressed as blue units per 0·2 cc. of 20 % solution.

Table I.

Trip No.	Weight of whole livers (lbs.)	No. of livers in lot	Variation in glycogen (g. per 100 g. liver)	Average glycogen (g. per 100 g. liver)	Oil (%)	Vitamin (blue units)
1	$< \frac{3}{4}$	17	Trace–7·47	1·57	16	269
	$> 1\frac{1}{2}$	10	Trace–4·78	1·71	18	1366
2	$< \frac{3}{4}$	21	0·04–0·90	0·45	18	386
	$> 1\frac{1}{2}$	8	0·05–0·79	0·33	20	1337

So great were the individual differences in glycogen values that in two later experiments a somewhat different procedure was followed. In place of storing the remainder of the livers in two large batches, each liver was stored separately until the glycogen values were obtained. The livers were then put into lots according to glycogen concentration. The results are given in Table II.

Table II.

Trip No.	Weight of whole livers (lbs.)	No. of livers in lot	Variation in glycogen (g. per 100 g. liver)	Average glycogen (g. per 100 g. liver)	Oil (%)	Vitamin (blue units)	Mean
3	$< \frac{3}{4}$	8	0·01–0·18	0·09	12·5	975	
		6	0·22–0·98	0·49	12·0	1469	1080
		6	1·58–3·27	2·2	18·1	797	
	$> 1\frac{1}{2}$	5	0·13–0·62	0·38	22	1742	1319
		3	1·48–1·82	1·67	22	897	
4	$< \frac{3}{4}$	8	Trace–0·56	0·27	20	283	
		4	0·72–1·67	1·12	18	699	407
		4	2·92–6·50	4·28	18	238	
	$> 1\frac{1}{2}$	4	Trace–0·27	0·13	25	663	
		3	0·79–1·02	0·94	18	550	583
		3	3·92–6·35	5·04	20	537	

The tables show clearly that there is no relation between the vitamin potency of the oil and the glycogen content of the liver.

For each experiment the mean value for vitamin A potency is lower for the small livers than for the large ones, this being further evidence in favour of the conclusions already arrived at [Lovern *et al.*, 1933] regarding age of fish as a factor influencing liver oil vitamin A potency.

SUMMARY.

(1) The diet of the halibut is of a general nature, with no outstandingly rich source of vitamin A to account for the high potency of halibut-liver oil.

(2) Taking the glycogen content of the liver as a criterion of intensity of feeding no correlation could be established between intensity of feeding and the vitamin A potency of the oil.

(3) Further evidence was obtained that in general the livers of older fish afford a more potent oil than those of younger fish.

REFERENCE.

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CXCIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXIV. A NOTE ON THE MECHANISM OF THE PRODUCTION OF PHENOLIC ACIDS FROM GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* DIERCKX.

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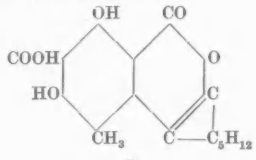
(*Received August 1st, 1933.*)

It was shown in Part XXIV of this series [Clutterbuck *et al.*, 1932] that when most species and strains in the *P. brevi-compactum* Dierckx series are grown on synthetic media containing glucose, or glucose and tartaric acid, as sole source or sources of carbon, five phenolic acids are present in the metabolism solution when all the glucose originally present has been utilised by the mould. The empirical formulae of these five acids are, in descending order of complexity, mycophenolic acid, $C_{17}H_{20}O_6$ (I), $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV), and 3:5-dihydroxyphthalic acid, $C_8H_6O_6$ (V). Their respective constitutional formulae have been either completely or almost completely elucidated [see Clutterbuck and Raistrick, 1933; Oxford and Raistrick, 1932; 1933].

Monomethyl ether of



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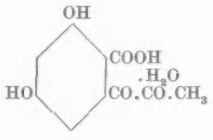

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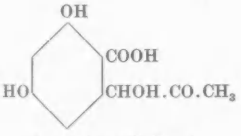

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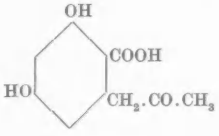

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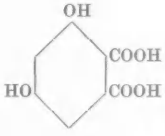

(V)

We may note here that all five acids are derivatives of resorcinol, and hence it seemed unlikely that all would prove to be stable end-products formed from glucose by five separate series of reactions, a more likely hypothesis being that some of these acids are intermediates in the formation of others. The present work was undertaken to discover the exact order of appearance of the phenolic metabolic products and the nature of the stable end-products of metabolism.

Experimental.

The strain of *P. brevi-compactum* Dierckx chosen (Catalogue Number M 3 (1)) was isolated by the late J. H. V. Charles in 1931 from mouldy Italian maize, and in that year gave relatively good yields of all five metabolic products when grown on Raulin-Thom medium [see Clutterbuck *et al.*, 1932, p. 1451]. 105 one-litre flasks each containing 350 cc. of Raulin-Thom medium of the following composition: glucose, 75 g.; tartaric acid, 4 g.; ammonium tartrate; 4 g. diammonium hydrogen phosphate, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4, 7H_2O$, 0·07 g.; $FeSO_4, 7H_2O$, 0·07 g.; water to 1500 cc., were sterilised and to each were added 3 cc. of a well-shaken suspension of spores in sterile distilled water (total volume, 350 cc.) prepared from 4 beer wort agar slopes of the above organism which had been incubated for 36 days. The flasks, after inoculation, were incubated at 24°, and rapid growth took place, a complete felt of mycelium being formed in every flask after 3 or 4 days' incubation. 26 flasks were removed after 8 days when the addition of ferric chloride to a test portion of the metabolism solution first showed definitely that phenols were present. The mycelium and the filtered metabolism solution were dealt with separately, the following tests and estimations being carried out on a small average sample of the latter: coloration with ferric chloride; approximate p_H; residual glucose by polarimeter; bromine absorption value in mg. per cc. (by Koppeschaar's method). The metabolism solution was then evaporated to 400 cc. *in vacuo* below 50° and worked up exactly as described by Clutterbuck *et al.*, 1932], except that the ether extraction at p_H 5·3 was omitted. The mycelium was dried, powdered and extracted in an all-glass Soxhlet apparatus first with boiling light petroleum (B.P. 50–60°) for 2 days, and then with ether for 2 days. It was found that when the mycelium contained mycophenolic acid, part of this acid was removed by light petroleum (in which it is very sparingly soluble) and crystallised out from the extract, the remainder being subsequently extracted by ether. The ethereal extract was evaporated, the residue was dissolved in a little alcohol and the mycophenolic acid isolated *via* the insoluble dipotassium salt.

The remaining flasks were taken off at the following intervals: 25 after 11 days; 20 after 15 days; 20 after 22 days (when all the glucose had been metabolised) and the remaining 14 after

Table I.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		Tests on metabolism solution					
Period of incubation (days)	Glucose metabolised (g.)	p_H	Bromine absorption value (mg. per cc.)	Coloration with ferric chloride	Tartaric acid recovered (g.)	Wt. of dried mycelium (g.)	Wt. of $C_{17}H_{20}O_6$ (I) (mycophenolic acid) (g.)
8	202 (54 %)	4	1·82	Not very intense brown	3·0	66	1·67 (63 % in mycelium)
11	269 (72·5 %)	4	2·80	More intense brown	0·8	83	2·28 (61 % in mycelium)
15	333 (90 %)	Between 4 and 5	3·86	Brownish-purple	0	87	2·67 (62 % in mycelium)
22	371 (100 %)	Between 4 and 5	4·64	Deep blue-crimson	0	78	2·85 (60 % in mycelium)
56	371 (100 %)	About 8	5·07	Deep blue-crimson	0	61	3·49 (all in metabolism solution; none in mycelium)

Notes: (*a*) Recrystallised from water, M.P. 120–132° (efferv.). Converted into 2:4-dinitrophenyl-
(*b*) The p_H 7 fraction weighed 3·3 g. of which 1·26 g. was $C_{17}H_{20}O_6$ (I). The rest (2·04 g.)

56 days. The last group of flasks was therefore incubated for 34 days after all the glucose had disappeared, the mycelial felts being partly waterlogged at the expiry of this period, and the metabolic products found in this final metabolism solution were, therefore, stable or relatively stable end-products of metabolism.

The results are summarised in Table I concerning which the following observations may be made.

(*a*) The data for each period of incubation are calculated on the basis of 20 flasks containing initially 371 g. glucose, 19 g. tartaric acid and 19 g. ammonium tartrate, the initial p_H of the medium being about 4.

(*b*) By tartaric acid recovered (column 6) is meant the weight of tartaric acid corresponding to the ether-insoluble part of the material precipitated during the evaporation of the metabolism solution to small bulk. This material gave strong reactions for potassium and for tartrates, and its very sparing solubility in water indicated that it consisted largely of potassium hydrogen tartrate.

(*c*) The weights of $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ recorded in columns 9, 10 and 12 were isolated from the final fraction extracted by ether at a reaction acid to Congo red, the total weight of this fraction being given in column 13. The much smaller fraction extracted at p_H 7 was worked up for mycophenolic acid only but undoubtedly contained small amounts of the C_{10} compounds. This unavoidable loss accounts in part for the discrepancy between the weights recorded in columns 14 and 15.

The following points arise from a consideration of the results in Table I.

(*a*) Utilisation of glucose and utilisation of tartaric acid by *P. brevi-compactum* take place concurrently. Although the presence of tartaric acid is necessary for good colour reactions in flasks it is not metabolised preferentially.

Table I (*cont.*).

(9)	(10)	(11)	(12)	(13)	(14)	(15)
Wt. of $C_{10}H_{10}O_6$ (III) (g.)	Wt. of $C_{10}H_{10}O_7$ (II) (g.)	Wt. of $C_{10}H_{10}O_5$ (IV)	Wt. of $C_8H_6O_6$ (V) (g.)	Wt. of "acid to Congo" fraction (g.)	Total wt. of crystalline phenols isolated (g.)	Total wt. of crude phenols from which (14) was isolated (g.)
2·41	Not detected with certainty but probably present	Trace detected with certainty, M.P. 146–150° efferv. remelts at 200–220°	Trace	3·56	4·08	6·0
3·69	0·13 (*a*)	Not looked for	0·03	5·79	6·13	9·32
4·05	1·3	Detected with certainty	Not worked up	7·97	8·02	12·42
3·65	3·0	None	0·11	10·55	9·61	14·31
None	5·14	None	0·30	10·20	8·93 (*b*)	15·73 (*b*)

hydrazone, M.P. 210–212° decomp. after crystallisation from ethyl acetate-light petroleum.
was necessarily lost in the estimation of $C_{17}H_{20}O_6$ (I).

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(*b*) For some reason at present unknown the ferric chloride coloration given by the metabolism solution in the early stages of growth is of no service in predicting which phenolic substances are present.

(*c*) As long as the p_H of the metabolism solution is well on the acid side of neutrality, the greater part of the mycophenolic acid remains in the mycelium. This is due to the fact that mycophenolic acid is almost insoluble in cold water but is readily soluble in dilute aqueous alkali. The other phenolic metabolic products are, on the other hand, very much more soluble in cold water.

(*d*) The stable end-products of metabolism are mycophenolic acid (I), and the acids $C_{10}H_{10}O_7$ (hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone II) and $C_8H_6O_6$ (3:5-dihydroxyphthalic acid V), but the final yield of the last named is small compared with the yields of the other two acids. The acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol III) is definitely an intermediate product of metabolism since its maximum yield (relatively very considerable) is reached on about the fifteenth day, after which, during the next 40 days, it is completely metabolised. The acid $C_{10}H_{10}O_5$ (3:5-dihydroxy-2-carboxybenzylmethylketone (IV)) is never at any time present in other than trifling amount and appears not to be a stable end-product of metabolism.

Discussion and Summary.

The results given in Table I definitely prove four series of facts, (*a*), (*b*), (*c*) and (*d*), and give fairly clear indication of another series (*e*).

(*a*) The yield of mycophenolic acid (I) increases continuously during the whole course of metabolism. A surprisingly large proportion, however (almost 50 %), of the total amount of mycophenolic acid (I) produced arises in the very early stages of metabolism (8 days after inoculation) and before growth of the organism is complete as is indicated by the weight of mycelium at this stage. Only a small proportion of the total yield of mycophenolic acid (less than 20 %) arises during the 34 days' incubation following the complete disappearance of glucose from the metabolism solution.

(*b*) The rate of production of the acid $C_{10}H_{10}O_7$ (II) is very different from that of mycophenolic acid. In the early stages of metabolism the presence of the acid $C_{10}H_{10}O_7$ could not be detected with certainty, and even after 11 days' incubation when 72·5 % of the glucose had been metabolised and growth of the mould had almost reached a maximum, only about 2·5 % of the final yield of the acid $C_{10}H_{10}O_7$ had been produced. On the other hand, about 40 % of the total amount of the acid $C_{10}H_{10}O_7$ produced arises towards the end of the incubation period, *i.e.* during the 34 days' incubation following the complete disappearance of glucose from the medium.

(*c*) The yield of the acid $C_{10}H_{10}O_6$ (III) increases rapidly to a maximum which is reached after an incubation period of 15 days, by which time 90 % of the glucose has disappeared, and then decreases in amount until this acid has disappeared completely from the medium after 56 days' incubation. Like mycophenolic acid (I) and unlike the acid $C_{10}H_{10}O_7$ (II) this acid is produced in largest amount (about 60 %) in the very early stages of metabolism, *i.e.* after 8 days' incubation.

(*d*) 3:5-Dihydroxyphthalic acid (V) is never present in large amounts but increases steadily during the whole course of metabolism, and at least 60 % of the total yield is produced after all the glucose has disappeared from the medium.

(*e*) The acid $C_{10}H_{10}O_5$ (IV) is present in very small amounts in the early stages of metabolism, but is absent in the later stages, none being detected after 22 days' incubation.

It must be admitted that the above results were entirely unexpected and are not easy of explanation, though it is hoped to obtain further light on the subject by a continuation of the work on somewhat different lines.

In view of the facts that mycophenolic acid (I), the acids $C_{10}H_{10}O_7$ (II), $C_{10}H_{10}O_6$ (III), $C_{10}H_{10}O_5$ (IV) and 3:5-dihydroxyphthalic acid (V) are all resorcinol derivatives, and that all have carbon side-chains in the 1 and 2 positions, it was natural to assume that one series of reactions or one set of precursors would be common to them all. The results presented do not offer conclusive evidence that this is the case.

We may, however, conclude with reasonable certainty that 3:5-dihydroxyphthalic acid (V) is an oxidation product of one or more of the other metabolic products.

Further, the fact that the acid $C_{10}H_{10}O_6$ (III) is produced in the early stages of growth and disappears completely in the later stages, while the acid $C_{10}H_{10}O_7$ (II) increases in amount throughout the whole course of metabolism and is produced in largest proportions during the later stages, renders it reasonably certain that the acid $C_{10}H_{10}O_7$ (II) arises by direct oxidation of previously formed $C_{10}H_{10}O_6$ (III). This change is indeed one which, as has already been shown [Oxford and Raistrick, 1933], can be readily brought about by purely chemical means. It is surprising to find that there should be a progressive accumulation of the acid $C_{10}H_{10}O_7$ (II), which is an α-diketone and which, certainly on purely chemical grounds, and probably on general biochemical grounds, might be expected to be readily oxidised by an organism whose available supplies of energy in the form of glucose have been exhausted.

The origin of the acid $C_{10}H_{10}O_5$ (IV) is not clear since the small yield and the difficulties of isolation render its estimation very difficult. It seems unlikely that the side-chain $—CH_2.CO.CH_3$ in this acid would be oxidised to $—CHOH.CO.CH_3$ in $C_{10}H_{10}O_6$ (III) more rapidly than the latter side-chain is oxidised to $—CO.CO.CH_3$ in $C_{10}H_{10}O_7$ (II), as must be the case if $C_{10}H_{10}O_5$ (IV) is the precursor of the other C_{10} acids. However, the ease with which it can be prepared *in vitro* from the acid $C_{10}H_{10}O_6$ (III) [Oxford and Raistrick, 1933] and the fact that it is present in the metabolism solution when the yield of the acid $C_{10}H_{10}O_6$ (III) is at a maximum, whereas it is absent when the acid $C_{10}H_{10}O_6$ (III) has disappeared and the yield of the acid $C_{10}H_{10}O_7$ (II) is at a maximum, indicate that it may be formed by the mould by reduction of the acid $C_{10}H_{10}O_6$ (III) and not by reduction of the acid $C_{10}H_{10}O_7$ (II).

It is tempting to postulate that the acid $C_{10}H_{10}O_6$ (III) is also the precursor of mycophenolic acid (I). The results presented do not exclude this possibility, but a consideration of the constitutional formulae assigned to these two acids shows that if this hypothesis is to be entertained certain assumptions must be made for which there are, so far as we are aware, no existing analogies in microbiological chemistry. Among other chemical changes necessary to convert $C_{10}H_{10}O_6$ (III) into mycophenolic acid (I) methyl and carboxyl side-chains must be introduced into the benzene nucleus. If, however, the possibility were admitted of introducing a —CHO group into the benzene nucleus by a microbiological process, then the introduction of two —CHO groups in the 4 and 6 positions into the molecule of the acid $C_{10}H_{10}O_6$ (3:5-dihydroxy-2-carboxyphenylacetylcarbinol, III) followed by the reduction of the —CHO group in the 6-position to CH_3, and the oxidation of the —CHO group in the 4-position to —COOH, immediately leads to the substituted aromatic nucleus shown to be present in mycophenolic acid (I).

The possibility that mycophenolic acid (I) is the precursor of the three

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C_{10}-acids, which might be supposed to be formed from it by oxidation, demethylation and decarboxylation, is rendered improbable by the fact that even after all the glucose has been metabolised the yields of both mycophenolic acid (I) and the acid $C_{10}H_{10}O_7$ (II) continue to increase. It must be remembered, however, that after the glucose has completely disappeared the mycelium decreases appreciably in weight, and hence the possibility cannot be ruled out entirely that the substances produced from the mycelium during this period might give rise to further amounts of mycophenolic acid (I) and/or the acid $C_{10}H_{10}O_7$ (II). Further, in view of the relatively large final yield of mycophenolic acid, this possibility would involve the assumption that, after all the glucose has been utilised, the mould loses to a very large extent its former power of oxidising mycophenolic acid.

Finally it must be pointed out that we have failed up to the present to find any indication as to what are the steps involved during the initial formation from glucose of the resorcinol nucleus common to all the five phenolic metabolic products of *P. brevi-compactum*, since investigation of the metabolism solution in the earliest stages of growth failed to reveal any products of metabolism other than the five phenolic metabolic products which we have described previously.

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CC. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XI. THE MEASUREMENT OF RESPIRATORY QUOTIENT, RESPIRATION AND GLYCOLYSIS WITH THE AID OF THE CONSTANT-VOLUME DIFFERENTIAL MANOMETER.

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The methods previously described in this series make possible the accurate measurement of the true respiratory quotient and respiration of tissues suspended in a phosphate medium [Dickens and Šimer, 1930], or in bicarbonate media (including serum) in equilibrium with CO_2 at physiological tension [Dickens and Šimer, 1931]. Additional points of technique were described recently [Dickens and Šimer, 1933]. These methods may be applied to all types of tissue; but when the bicarbonate method is applied to tissues in which the aerobic acid production is high compared with the respiration, the glycolysis may not persist throughout the experiment at its initial level. For with such tissues, although it is adequate with others, the amount of bicarbonate solution which can be used may be unable to prevent an undue fall of p_H and increase of lactate concentration in the medium. With the simple manometer used, and also with the Barcroft manometer as applied to the measurement of respiratory quotient by Dixon and Keilin [1933], the permissible bicarbonate reserve is limited by the length of the manometer limb and the density of the manometer fluid. Increase of the former is inconvenient; increase in the latter results in loss of sensitivity. The difficulty has been overcome by the application of the constant-volume differential manometer [Dickens and Greville, 1933] to the respiratory measurements. It is now possible to use a sensitive manometer fluid (Brodie fluid of sp. gr. approximately 1), and to have a volume of bicarbonate solution such that, even with highly glycolysing tissues, the glycolysis remains nearly constant throughout. The conditions are strictly comparable with those of the Warburg [1924] two-vessel method; but whereas Warburg's method gives only the O_2 consumed and the total CO_2 produced by respiration and glycolysis, the method now described gives the true respiratory quotient and the true aerobic glycolysis, respiratory CO_2 and O_2-uptake.

Method.

Principle. The constant-volume differential manometer has both vessels rigidly attached to manometer limbs which may be moved independently up and down. By this means the meniscus in each limb is kept at a fixed point in the

scale, and the contents of the vessel at constant volume; the difference in pressure between the vessels is given in terms of height of manometer fluid by the vertical distance between the menisci in the two limbs. Each limb has a tap which enables the vessels, the limbs and the outside air to be connected in pairs or all together. When this manometer is used for the measurement of R.Q., equal weights of tissue are put into equal volumes of medium in the two vessels, which are of equal size. Excess of acid is tipped into the medium in one vessel at the beginning of the experiment, in the other at the end. The pressure difference between the two vessels immediately before addition of acid to the medium in the first vessel is zero; after acid addition in the second vessel it is equal to the pressure increase due to the respiratory CO_2 less the pressure decrease due to the absorbed O_2. The total CO_2 in both vessels is now absorbed, when the pressure difference between them represents the O_2 absorbed during the experiment. This subtracted from the previous pressure difference gives the respiratory CO_2, so that the R.Q. can at once be calculated. Hence, the measurement of respiration and R.Q. necessitates only two readings; that of aerobic glycolysis, which is deduced from the pressure change in the second vessel during the experiment, demands a third reading in conjunction with a thermobarometer. The latter is unnecessary if only R.Q. and respiration are to be measured.

Apparatus. The constant-volume differential manometer, its use including filling with gas and its calibration have already been fully described [Dickens and Greville, 1933]. For the measurement of R.Q. the only modification lies in the use of a special vessel of new design[1] (Fig. 1). This is of conical shape and of

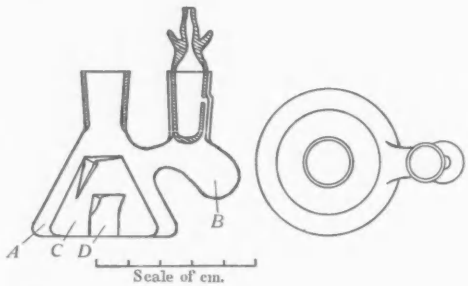

Fig. 1.

20–25 cc. volume. The diameter of the base should be about equal to the height: this gives stability to the vessel and large surface to the solutions. The vessel contains a separate inner conical part *C*, in the centre of the base of which there is a small cylindrical vessel *D* with a lip, like a small jug. *D* and *C* have each a groove at a suitable point in their rim to facilitate the insertion of pipettes and tissue. *B* is a bulb communicating with the outer part *A*; it has a bored stopper which can be closed by turning [Warburg and Kubowitz, 1929] and which is provided with hooks from which rubber bands may be stretched to hooks on the vessel. On inclining the vessel to the left (Fig. 1), liquid may be tipped from *B* into *A*; on inclining to the right, liquid first runs from *A* into *B*, and finally the contents of *D* drain almost completely into *C*.

[1] Made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

Having only one stopper, the vessel is more convenient and less likely to leak than that described for the original bicarbonate method [Dickens and Šimer, 1931]; it is also more stable; hence its use in that method is now recommended.

Procedure. The solutions are first measured into the various parts of the vessels as follows:

A. Bicarbonate-containing medium (3·0 cc. are usually taken).

B. 2·5 *N* hydrochloric acid, 0·25 cc.

C. 43 % NaI, $2H_2O$, 2 cc. (acidified immediately before experiment with 0·1 *N* sulphuric acid, methyl orange as indicator).

D. 30 % $NaMnO_4$, $3H_2O$ containing *N*/400 H_2SO_4, 0·5 cc.

The medium must be accurately measured with a grease-free pipette with two marks and a fine tube; such a pipette delivered 3 cc. with an extreme error of ± 4 mg. in twelve consecutive measurements. After the solutions have been measured, the stoppers of the vessels, greased with a good rubber lubricant (Stevens's rubber grease), are worked in by turning. The tissue is then prepared for the experiment. In order to equalise as nearly as possible the conditions of the two pieces of tissue, the portions to be used are suspended for 7 minutes in bicarbonate-containing medium through which oxygen containing 5 % carbon dioxide is passing and are then rinsed for a further 3 minutes in oxygenated bicarbonate-free salt solution. Portions are then weighed on a torsion balance after draining on filter-paper, the weights for each vessel being equal to the nearest mg., and are inserted in the medium in the spaces *A*. The vessels are attached to the manometer by the ground joints lubricated with rubber-grease, and placed in the bath. The gas stream (5 % CO_2 in O_2), passing first through a small water-containing gas-bubbler immersed in the bath and then through a cotton-wool plug, enters the apparatus through the upper tubes of the manometers and leaves through the stoppers of the vessels below the water-level in the bath. Extra gas-pressure should be applied by means of a screw-clip or tap on the safety device [Dickens and Greville, 1933, Fig. 3]. After the gas has passed for 10 minutes, during which the manometer has been continuously shaken, the extra pressure is released, the stoppers of the vessels turned and worked in until they will no longer move and the manometer shaken for a further 2 minutes with the vessels open to the air through the taps. The shaking is then interrupted, the vessels shut to the air and connected to the limbs, the menisci in the limbs being level and at the centre (150) mark. The right-hand tap is turned so that the limb is open to the air and the vessel closed: the acid is then tipped from the bulb into the medium in the right-hand vessel, the limb being temporarily taken out of the clips. After the right-hand vessel has been replaced, shaking is resumed. If glycolysis is to be measured, a simple manometer should have been placed in the bath at the same time as the differential manometer, and readings of both manometers are taken at intervals until the end of the experiment. When the differential manometer is used as a simple manometer, the two menisci are still kept to their appropriate marks, the position of the mark on the left-hand limb being read off as usual on the graduated scale on the right-hand limb.

At the end of the experimental period (usually 2 hours), during which 120–200 $mm.^3$ oxygen should have been consumed, a final reading for use in calculation of glycolysis is taken, the meniscus in the left-hand limb is brought to the mark, the left-hand tap is turned so that the vessel is closed, and the acid is

tipped from the bulb. After shaking has been continued for a few minutes, both menisci are levelled at their marks, the limbs being open to the air. Both limbs are put in communication with their vessels and closed to the air, and 10 minutes after adding the acid a reading is taken, the constancy of which is checked 5 minutes later. This reading less 150 = H_1. The permanganate is tipped into the iodide in both vessels and the manometer shaken until constancy of the reading shows that gas absorption is complete. This usually requires 35 minutes, but longer times, up to 1 hour, are occasionally necessary. The final reading less 150 = H_2.

Calculation. Let h_{O_2} = pressure-change caused by consumption of oxygen and h_{CO_2} = pressure change due to respiratory carbon dioxide.

All pressures are reckoned algebraically in terms of mm. of Brodie fluid. With the procedure described, h_{O_2} is always negative, h_{CO_2} is always positive, whilst gas evolution in the left-hand (experimental) vessel always causes an increase in the reading.

After complete liberation of the bound carbon dioxide in both vessels, equal amounts of bound carbon dioxide having been present at the beginning of the experiment, any difference of pressure between the vessels is due only to the respiratory exchange during the experimental period.

Hence $$H_1 = h_{O_2} + h_{CO_2} \qquad \text{.....(1).}$$

After complete absorption of the carbon dioxide in the two vessels, the pressure difference is due to the oxygen consumed in the experimental vessel during the experimental period.

$$H_2 = h_{O_2} \qquad \text{.....(2).}$$

Subtracting, $h_{CO_2} = H_1 - H_2$, and, as usual,

$$x_{O_2} = H_2 . k_{O_2}; \quad x_{CO_2} = (H_1 - H_2) . k_{CO_2},$$

where k_{O_2} and k_{CO_2} are the simple manometer vessel-constants [Warburg, 1926].

Hence, $$\text{R.Q.} = -\frac{x_{CO_2}}{x_{O_2}} = \left(1 - \frac{H_1}{H_2}\right) \frac{k_{CO_2}}{k_{O_2}} \qquad \text{.....(3).}$$

Now let h = pressure change read off on the instrument during the period in which it is used as a simple manometer, corrected for thermobarometer changes.

Then $h = h_{O_2} + h_{CO_2} + h_b$ where h_b = pressure-change due to alteration in the amount of bound CO_2 during the experimental period. By (1), $h_b = h - H_1$, so that

$$x_b = (h - H_1)\, k_{CO_2} \qquad \text{.....(4).}$$

Table I.

Both vessels contained:

In *A*, 3 cc. bicarbonate solution, containing *ca.* 1700 mm.³ bound CO_2.
In *B*, 0·3 cc. 2·5 *N* HCl.
In *C*, 2·0 cc. 43 % sodium iodide solution.
In *D*, 0·5 cc. 30 % sodium permanganate solution.

Filled with gas mixture containing 5 % carbon dioxide.

Pressure change on acidification of both vessels (corresponding to error in H_1):

+1, 0, +1, 0, +0·5, 0, −0·5 mm. Brodie fluid.

Pressure change on acidification followed by CO_2 absorption in both vessels (corresponding to error in H_2):

+1, +2, 0, 0·5, 0, 0 mm. Brodie fluid.

Table II.

Tissue	Moist wt. in each vessel (mg.)	Bicarbonate concentration (mM/litre)	Pressure difference (mm. Brodie fluid) After acidification	Pressure difference (mm. Brodie fluid) After absorption
Rat testis	100	26	0	−1·5
Rat kidney	50	26	0	−1
Jensen rat sarcoma	95	26	−1	−3
,,	100	26	−1	−1
,,	100	34	−1·5	−1
,,	100	34	−2	0

Protocols.

The protocols show results with examples of two types of tissue, (1) a normal tissue of known R.Q. = 1, with little aerobic glycolysis, and (2) a tumour tissue with high aerobic glycolysis and low R.Q.

(1) *Rat brain.*

Thin slices of cerebral cortex; rinsed 5 mins. in bicarbonate-Ringer in stream of $O_2 + 5\%$ CO_2, 5 mins. in salt solution in stream of O_2, before experiment.

Gas mixture, O_2 with 5·46 % CO_2. Bicarbonate content of Ringer 530 mm.^{3} CO_2/cc.

Glucose 0·2 %. 37·2°. p_H 7·4.

Differential manometer filled with Brodie fluid: vessel vols. 23·23 cc. (both).

Contents of vessels:

A, 3·0 cc. bicarbonate-Ringer-glucose solution + tissue.
B, 0·25 cc. 2·5 *N* HCl.
C, 2·0 cc. 43 % sodium iodide solution.
D, 0·5 cc. 30 % sodium permanganate solution.

Vol. fluid 5·75 cc. Vessel consts. $k_{O_2} = 1{\cdot}55$, $k_{CO_2} = 1{\cdot}84$.

Experiment:

Differential manometer. Pressure readings (mm. Brodie fluid).

Time (mins.)		
0	150	
	———	Acidify closed R.H. vessel.
2	150	
		$h = +3$ (corrected for thermobarometer).
120	156	
	———	Close L.H. vessel and acidify. Level at 150. Open both vessels to manometer.
130	136·5	
135	137	
	———	$H_1 = -13$. Add $NaMnO_4$ to NaI, both vessels.
170	70	
175	69·5	$H_2 = -80$.
185	70	

Weight of tissue: wet (L.H.) 60 mg.; (R.H.) 60 mg. dry (L.H.) 5·5 mg.; (R.H.) 6·3 mg.

Calculation:

Time of experiment: 2 hrs. 0 min.

$H_2 = h_{O_2} = -80$ mm. $x_{O_2} = -124$ mm.^{3}

$h_{CO_2} = H_1 - H_2 = +67$ mm. $x_{CO_2} = +123$ mm.^{3}

$h_b = h - H_1 = +16$ mm. $x_b = +29$ mm.^{3}

Whence $Q_{O_2} = -11{\cdot}3$ (mm.^{3} O_2 per mg. dry tissue per hour).

$Q_M^{O_2} = +2{\cdot}6$ (mm.^{3} acid produced per mg. dry tissue per hour).

R.Q. = 0·99.

(2) *Jensen rat sarcoma.*

11-day tumour; thin slices; pre-treatment, vessels, vessel-constants, gas mixture and solutions, as for brain (1), except that bicarbonate content of Ringer solution = 666 mm.³/cc.; hence p_H (initial) = 7·5; (final) = 7·3. 37·2°.

Experiment:

Differential manometer.

Time (mins.)		
0	Level at 150 mm. and acidify closed R.H. vessel.	
30	+ 20·5	Pressure changes in 30-min. periods corrected for thermobarometer.
60	+ 22·5	
90	+ 25	
120	+ 27	
150	+ 27	
	h = +122	Close L.H. vessel and acidify. Level menisci at 150 mm. Open both vessels to manometer.
	Pressure readings	
160	109	
165	110	H_1 = −40. Add $NaMnO_4$ to NaI, both vessels.
210	36	
220	32·5	
235	32·5	H_2 = −117·5.

Weights of tissue { wet (L.H.) 55 mg.; (R.H.) 55 mg. / dry (L.H.) 5·3 mg.; (R.H.) 6·2 mg.

Calculation:

Time of experiment 2 hrs. 30 mins.

$$H_2 = h_{O_2} = -117{\cdot}5 \text{ mm.} \quad x_{O_2} = -182 \text{ mm.}^3$$
$$h_{CO_2} = H_1 - H_2 = +77{\cdot}5 \text{ mm.} \quad x_{CO_2} = +142{\cdot}5 \text{ mm.}^3$$
$$h_b = h - H = +162 \text{ mm.} \quad x_b = +298 \text{ mm.}^3$$

Whence $Q_{O_2} = -13{\cdot}7$.

$Q_M^{O_2} = +22{\cdot}4$.

R.Q. = 0·78.

Simultaneously made experiments on slices of the same tumour suspended in 1·5 cc. bicarbonate-Ringer by Dickens and Šimer [1931] method gave R.Q. = 0·81, $Q_{O_2} = -12{\cdot}7$; whilst a determination of aerobic glycolysis by Warburg's 2-vessel method gave $Q_M^{O_2} = +22{\cdot}3$, if the value of R.Q. found by the constant-volume differential manometer experiment given above be assumed for the calculation.

Notes.

(1) *Absorption of carbon dioxide.* The production of an absorbent for carbon dioxide by the mixing of two solutions, such as NaI and $KMnO_4$, avoids the necessity of having taps for the introduction of alkali into the vessel during the experiment. The quantities of the reagents used for carbon dioxide absorption by Dickens and Šimer [1931] are insufficient for the present method; more concentrated solutions are used, and it is necessary to use the more soluble sodium permanganate. The pressure change accompanying the mixing of iodide and permanganate which has no relation to CO_2 absorption may become large with concentrated solutions. It is probably due to alterations either of vapour pressure or of volume. The pressure changes observed on adding 30 % permanganate to sodium iodide solutions of various concentrations are shown in Fig. 2, from which it is seen that by choosing suitable concentrations the pressure change on mixing strong solutions may be reduced below that found with the original dilute solutions. We have therefore used in all experiments 30 % $NaMnO_4$, $3H_2O$ and 43 % NaI, $2H_2O$, 0·5 cc. of the former being added to 2 cc. of the latter. Carbon dioxide absorption by these quantities is rapid and complete. Since they were found to absorb completely 6·4 cc. carbon dioxide, *i.e.* 25 % of the vessel volume and twice as much carbon dioxide as is likely to be in the vessel during a R.Q. determination, it is clear that an ample excess of alkali is produced.

(2) *Accuracy.* In the absence of tissue, errors may be introduced by inaccurate measurement of the bicarbonate solutions and unequal filling of the two vessels with gas. The magnitude of the errors occurring in absence of tissue with careful measurement and technique as described is shown by Table I. When equal wet weights of tissue are inserted into the two vessels, the accuracy is limited by the agreement in their initial bicarbonate contents and in their glycolyses during the preliminary period. In the control experiments in Table II amounts of tissue suitable for R.Q. determinations were prepared and the vessels filled by the technique described, but 2 minutes

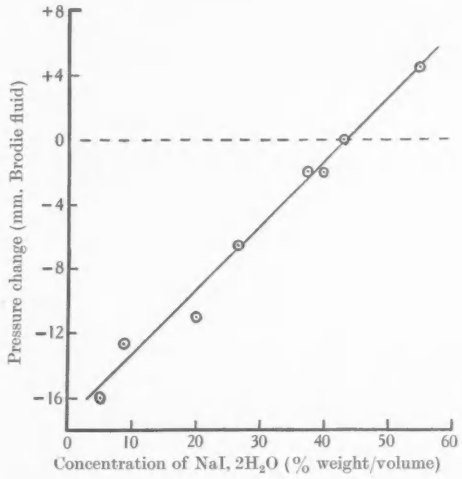

Fig. 2. Pressure changes on adding 0·5 cc. 30% $NaMnO_4$, $3H_2O$ to 2 cc. of sodium iodide solutions of various concentrations; 37·3°; air in gas space. 0·2 cc. *N* NaOH in vessel as CO_2 absorbent. Permanganate solution contained $N/400$ H_2SO_4, iodide contained $N/100$ H_2SO_4.

after stopping the gas stream the acid was tipped from the bulbs into both vessels. After a steady reading had been obtained, the carbon dioxide was absorbed in both vessels. The figures in the fourth column correspond to errors in H_1, those in the fifth to errors in H_2. In the last two experiments the strong bicarbonate-Ringer solution now used for tumour experiments (see Protocol) was taken. H_2 is always much larger than H_1, and errors of a few mm. in H_2 have a negligible effect on the R.Q. The possible error in the latter, governed by the error in H_1, with a respiration corresponding to 100 mm. Brodie fluid, is $\pm 2{\cdot}5$ %. This is equal to the error of the Dickens and Šimer method [1931]; but it is to be noted that in the present method the necessary respiration is only one-half and the bicarbonate reserve over twice that obtaining in the earlier one.

(3) Any oxygen uptake or carbon dioxide production during the experimental period by the tissue in the vessel in which the acid is added from the bulb at the beginning of the experiment will lead to faulty values of x_{O_2} and x_{CO_2}. The significance of oxygen uptake by acidified tissue in the measurement of R.Q. is discussed by Needham [1932, 1, 2]. We have found, however, that various types of tissue, when acidified under conditions comparable with those of the acidified tissue in the present method, show an oxygen uptake always less than 1 % of that of the non-acidified tissue. Further, in absence of carbon dioxide absorbent, such acidified tissue causes no pressure change. It is therefore concluded that the presence of acidified tissue in this method has a negligible effect on the observed value of the R.Q.

If at the beginning of the experiment, the metabolism of the tissue in one vessel could be immediately and completely stopped without altering the acid-base equilibrium, the presence of acidified tissue during the experimental period would be avoided, and also there would be no need

for a thermobarometer to assist in the measurement of h. The method would thereby be improved, but no suitable agent for killing the tissue has yet been found.

(4) This method has been developed during the last two years independently of that described recently by Dixon and Keilin [1933], which is essentially an application of the principle of the Dickens and Šimer [1931] method to the Barcroft manometer. Not only has the application of the constant-volume differential manometer, in preference to the Barcroft instrument, made possible a method which combines an increased sensitivity with a larger bicarbonate reserve, but also the flexibility of the rubber connection between the two limbs allows the use of rigidly fixed vessels without the necessity for ground-in bulbs and for the introduction of extraneous fluids by taps attached to the vessel. It is our experience that only stoppers which have been worked in until they will no longer turn are consistently capable of withstanding without danger of leak the large pressure differences involved in the determination of a respiratory quotient.

Summary.

The constant-volume differential manometer previously described by the authors has been applied to the measurement of respiratory quotient, respiration and glycolysis in bicarbonate media, with the help of the principle used by Dickens and Šimer [1931] in the earlier method for this purpose. Advantages of the new method are:

1. It is not necessary to use dense manometric fluids.
2. The sensitivity is increased above that of the previous method, so that for the same accuracy less tissue and a shorter experimental period may be used.
3. At the same time the bicarbonate reserve in the vessels may be greatly increased. This enables accurate measurement of the aerobic acid production of highly glycolysing tissue, for which the previous method gave low values.
4. The conditions for measurement of glycolysis are comparable with those in the Warburg two-vessel method; but the new method, like the other two described in this series, has the great advantage that the metabolism is measured on a single piece of tissue.

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CCI. THE ABSORPTION OF *n*-HEXADECANE FROM THE ALIMENTARY TRACT OF THE RAT.

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The degree to which unsaponifiable substances are absorbed from the alimentary tract has assumed considerable importance in the last few years on account of the biological significance of the fat-soluble vitamins and carotene. In previous papers the absorption of squalene, phytol, oleyl and cetyl alcohols, cholesterol and a medicinal liquid paraffin has been investigated [Channon, 1926; Channon and Collinson, 1928; 1929]. In the last case, it was found that the liquid paraffin was absorbed to some extent by the rat. Further, from consideration of the cholesterol content of the liver unsaponifiable fractions and of the iodine values of the non-sterol portion of those fractions, it was deduced that the liquid paraffin was present in the liver. By further experiments on pigs, it was found possible chemically to isolate the paraffin from the liver of that animal. These results were of interest because firstly they demonstrated the absorption of hydrocarbons from the alimentary tract to a limited extent at least, even though previous investigations had led to the current view that such hydrocarbons were not absorbed, and secondly they focused attention on the apparent rôle of the liver in storing unsaponifiable substances generally. Demonstration of the former fact raised the question as to the mechanism whereby such inert substances as hydrocarbons are absorbed. Since chemical investigation of the liquid paraffin used showed that it consisted of a mixture of polycyclic hydrocarbons, it was felt that the positive results obtained for its absorption, which seemed at variance with previous work, might be attributable to its particular chemical nature. For this reason, it was deemed necessary to carry out further work using a synthetic normal hydrocarbon.

Experimental.

Preparation of hexadecane.

n-Hexadecane was chosen for this work because it can be prepared readily from palmitic acid and secondly, the fact that it is liquid at room temperature (m.p. 18°) should facilitate its absorption. Pure ethyl palmitate was prepared either from palm oil or commercial palmitic acid. In the main preparation 348 g. of ester dissolved in the minimum volume of warm alcohol (which had been previously distilled after dissolution in it of 2 % of sodium and then boiled for a day with metallic calcium and again distilled) were slowly run on to 209 g. of sodium at 130°. The cetyl alcohol isolated weighed 280 g. (94 %

of theory). The alcohol was converted into the iodide by means of red phosphorus and iodine and the iodide converted into *n*-hexadecane by treatment with zinc dust in glacial acetic acid into which hydrochloric acid gas was occasionally bubbled for a few minutes. The hexadecane was then distilled. The first preparation (B.P. 287°, M.P. 18°) gave C 84·48, H 14·68 % (calc. C 84·85, H 15·15 %) and this was used for exp. I. For exp. II, part of this material was again distilled and the product used gave C 84·60, H 15·1 %. For exp. III, the large preparation after purification with concentrated sulphuric acid at 130° gave C 84·81, H 15·15 %.

Animal experiments.

In each of exps. I and II, two groups of rats, containing the same number of animals of each sex, were fed on a synthetic diet (caseinogen 20, butter 10, starch 60, marmite 5, salt mixture 5) for 21 and 20 days respectively. The experimental groups received in addition, immediately before feeding, doses of hydrocarbon delivered into the mouth from a micro-burette, the dose being gradually increased from 0·035 to 0·12 cc. daily. The animals were then killed by chloroform after being without food for 24 hours. Their livers were removed and the unsaponifiable fractions prepared as described by Channon [1926].

The faeces had been collected twice weekly and stored under absolute alcohol and at the close of the experiments the cages and trays were rinsed with ether, which was added to the main extracts. The faeces from each batch of rats were then boiled separately with a litre of absolute alcohol. This alcohol extraction was repeated three times and followed by three extractions with ether under reflux. The finely powdered residues were then further treated with ether for 12 hours in a Soxhlet extractor. After evaporation to dryness of the combined alcohol-ether extracts, the material was saponified with strong alcoholic potassium hydroxide and the unsaponified material extracted by ether. After a second saponification of this material, the faecal unsaponifiable fraction was obtained. Cholesterol was determined by the digitonin method and the iodine value by the Rosenmund and Kuhnhenn [1923] methods on the unsaponifiable fractions from both livers and faeces.

The results are recorded in Table I.

Table I.

Exps. I and II.

	I	II
Duration of experiment (days)	20	21
Amount of hexadecane administered (g.)	12	14

	Animals receiving the control diet		Animals receiving control diet *plus* hexadecane	
	I	II	I	II
The unsaponifiable matter of the livers				
No. of animals	9	10	9	10
Wt. of livers (g.)	69·4	77·2	79·9	82·3
(*a*) Wt. of unsap. fraction (g.)	0·226	0·320	0·238	0·3835
Wt. of sterol in (*a*) (g.)	0·162	0·150	0·190	0·1788
(*b*) Wt. of non-sterol fraction of (*a*) (g.)	0·064	0·170	0·048	0·2047
Iodine value of (*a*)	94·7	87·3	89·1	90·7
The unsaponifiable matter from the faeces				
(*a*) Wt. of unsap. fraction (g.)	2·60	2·26	5·80	6·40
Iodine value of fraction	69·0	62·6	32·8	30·1
Wt. of sterol in unsap. fraction (g.)	0·78	0·72	1·00	1·02
Wt. of non-sterol fraction of (*a*) (g.)	1·82	1·54	4·80	5·62

The results are rendered more lucid by the figures in Table II in which are recorded the yields of unsaponifiable material and sterol per 100 g. of liver, together with the iodine values of the non-sterol fraction calculated from the cholesterol content.

Table II. *Yields of unsaponifiable matter and cholesterol mg./100 g. of liver.*

Exp. I	Unsap. matter	Sterol	Non-sterol	Iodine value of non-sterol fraction
Control	325	233	92	169
Control + hexadecane	298	238	60	181
Exp. II				
Control	415	194	221	106
Control + hexadecane	466	217	249	111

The mean values calculated from 11 control groups of animals in results reported by Channon [1926] and Channon and Collinson [1928] in similar experiments are: unsap. matter 414, cholesterol 296, non-sterol 118 mg. per 100 g. liver, and the figures recorded in Table II all lie within the range of normal variation in those 11 experiments.

Thus the administration of the hydrocarbon has had no significant effect on the yield of the unsaponifiable material of the livers, or on its sterol content. Further, the iodine values of the non-sterol part of the unsaponifiable fractions show no significant difference between the control and experimental animals. With these figures may be contrasted the results of the two experiments of Channon and Collinson [1929] with the polycyclic hydrocarbon mixture. As a mean value for two experiments, these authors found the significant result that in the control animals the material other than sterol in the unsaponifiable fraction constituted but 22·7 % of the whole and had an iodine value of 118·8, as against 56·2 % and 30·8 for the corresponding figures for the animals which received the polycyclic paraffin. It is to be noted however that they too observed no increase in the actual amount of the unsaponifiable matter in the livers.

The unsaponifiable matter of the faeces.

From the figures recorded in Table I on the unsaponifiable fraction prepared from the faeces, certain deductions may be made. In exp. I the weight of the non-sterol fraction in the control group is 1·82 g., and in the faeces of the animals which received the hydrocarbon, 4·80 g. The difference between these two values, namely 2·98 g., gives an approximate value for the amount of the hydrocarbon excreted. Further, an approximate check on this value may be obtained from calculations made from the iodine values of the unsaponifiable fractions and their sterol content, provided it be remembered that the use of the iodine value of cholesterol, 65·8, for the mixture of sterols present in the faeces is arbitrary. Calculation on this basis shows in the control group of exp. I that the fraction of the unsaponifiable matter which is not sterol, 1·82 g., has an iodine value of 70·4, whilst the corresponding fraction for the animals fed with hexadecane, 4·80 g., has an iodine value of 25·9. If the increased amount of the faecal unsaponifiable matter in the latter group is due to the presence of hexadecane, of iodine value zero, and if that part of the unsaponifiable matter which is neither sterol nor hydrocarbon has the same iodine value as the corresponding fraction from the control group, namely 70·4, the amount of the latter may be calculated. The figure so obtained for the amount of hexadecane in the

faecal material is 4·80 – 1·75 = 3·05 g. which agrees well with the figure obtained directly by difference between the weights of the non-sterol fractions, *i.e.* 2·98 g. Therefore in exp. I, of 12 g. of hexadecane administered, 3 g. only have been excreted. Similar calculations may be applied to exp. II, and the combined results are set out in Table III.

Table III. *Excretion of hexadecane in the faeces.*

	Animals receiving control diet		Animals receiving control diet *plus* hexadecane	
	Exp. I	Exp. II	Exp. I	Exp. II
(*a*) Wt. of non-sterol fraction (g.)	1·82	1·54	4·80	5·62
(*b*) Iodine value of non-sterol fraction (calc.)	70·4	61·2	26·3	22·3
(*c*) Wt. of material other than hydrocarbon in (*a*) (g.) (calc. from (*b*)	—	—	1·75	2·05
(*d*) Wt. of hydrocarbon (g.):				
(1) By difference from (*a*)	4·80 – 1·82 = 2·98		5·62 – 1·54 = 4·08	
(2) By calc. from (*b*)	4·80 – 1·75 = 3·05		5·62 – 2·05 = 3·57	
	Exp. I		Exp. II	
(*e*) Wt. of hexadecane (g.) (Mean values of (*d*) 1 and (*d*) 2)	3·00		3·83	
Hexadecane administered (g.)	12·00		14·00	
Hexadecane not recovered (g.)	9·00		10·17	

In the two experiments therefore 9·0 and 10·17 g. of the hexadecane administered were not recovered in the faeces. This corresponds with an absorption of 50 and 49 mg. per rat per day respectively. Hence, although there has been no alteration in the amount or character of the unsaponifiable matter of the livers, a definite amount of the hydrocarbon has been absorbed. In view of the fact that Channon [1926] isolated squalene from the livers of rats which had received squalene in their diet, and that Channon and Collinson isolated the polycyclic hydrocarbon from the liver of a pig which had received liquid paraffin, the failure of the hydrocarbon to appear in the liver is unexpected. Since the liver seems to fulfil the rôle of storing unsaponifiable substances, the absence of hexadecane suggested that the 19 g. absorbed by 19 rats in 21 days may have been completely oxidised or less probably stored in part elsewhere. It was accordingly decided to carry out a further experiment more closely akin to the conditions of that of Channon and Collinson [1929] and at the end to attempt to determine whether the hydrocarbon were present not only in the livers but also in the carcases. In this experiment, the hydrocarbon was incorporated in a similar diet to the extent of 4 % for 15 days which was increased to 6 % for a further 9 days, the percentage of starch being correspondingly reduced. Every care was taken to keep the experiment as quantitative as possible and the following method was adopted for incorporating the hexadecane in the diet: one-half the butter required for a given quantity of diet was melted and run in to a weighed amount of hydrocarbon in another vessel. After mixing, the butter-hydrocarbon mixture was incorporated in the dry diet, the remainder of the butter being used successively to rinse out the vessel in which the hexadecane and butter had been mixed.

The unsaponifiable fractions of the livers and faeces were prepared as in the previous experiments, and after removal of the entire alimentary tract the carcases were also worked up. They were warmed on a water-bath with 5 % aqueous sodium hydroxide until digestion was complete, when the fat was exhaustively extracted by ether. The fat was then resaponified with alcoholic

potash and the unsaponified material obtained. In view of the large amount of fat present prior to the second saponification, the resultant product was again submitted to saponification with alcoholic sodium ethoxide. The results of exp. III are set out in Table IV.

Table IV.

Exp. III.

Duration of experiment 25 days. Hexadecane administered 149 g.

	Animals receiving the control diet	Animals receiving the control diet *plus* hexadecane
The unsaponifiable matter of the livers		
No. of animals	13	13
Wt. of livers (g.)	107·2	111·0
Wt. of unsap. fraction (g.)	0·3403	0·3276
Wt. of sterol in unsap. fraction (g.)	0·2327	0·2402
Iodine value of unsap. fraction	83·3	65·8
Wt. of material other than sterol (g.)	0·1076	0·0874
The unsaponifiable matter of the carcases after removal of the alimentary tract and liver		
Wt. of unsap. fraction (g.)	5·01	8·04
Wt. of sterol in unsap. fraction (g.)	2·68 (53·5 %)	3·63 (45·1 %)
Iodine value of unsap. fraction	73·8	58·2
Wt. of material other than sterol (g.)	2·33	4·41
The unsaponifiable matter of the faeces		
Wt. of unsap. fraction (g.)	3·90	118·3
Iodine value of unsap. fraction	53·7	9·4

The results calculated on a percentage of the weight of liver are recorded in Table V.

Table V. *Yields of unsaponifiable matter and cholesterol mg./100 g. of liver.*

	Unsap. matter	Sterol	Non-sterol	Iodine value of non-sterol
Control diet	317	217	100	122
Control diet + hydrocarbon	295	216	79	66

These figures also show no increase in the amount of the unsaponifiable matter in the livers of the experimental group although the iodine value of the non-sterol fraction is 66 compared with 122 for that of the control animals, a result similar to that of Channon and Collinson, previously mentioned. The amount of hydrocarbon in the faeces, about 114 g., leaves 35 g. unaccounted for. This corresponds to a daily absorption per rat of 108 mg. an amount rather more than twice as great as was obtained in exps. I and II, a result to be anticipated because in those experiments the animals received the hexadecane in drops before being fed in the morning, while in exp. III the considerably greater amount of hydrocarbon administered was dissolved in the butter before admixture with the rest of the diet.

On the other hand, although increase in the amount of unsaponifiable matter of the livers has occurred, study of the figures recorded in Table IV reveals the fact that the amount of unsaponifiable matter yielded by the carcases of the experimental animals, 8·04 g., is some 60 % greater than that of those of the control group, 5·01 g.

Further the non-sterol fraction present is 4·41 g. compared with 2·33 g. of the control group. Assuming that the increase is due to the presence of hexadecane only, simple subtraction suggests the presence of 2·08 g. of that substance.

Further, if the iodine value of the material which is not sterol or hydrocarbon in the unsaponifiable fraction from the animals which received hexadecane is the same as that of the non-sterol material from the control group, calculation shows that the former must contain 1·65 g. of a saturated substance. This figure is in fair agreement with that obtained by simple subtraction, 2·08 g. The presumptive evidence of the presence of the hydrocarbon in the carcases was strengthened by the semi-liquid appearance of the unsaponifiable fraction from the experimental group and its low acetyl value, but it was not finally confirmed by chemical isolation of the hexadecane. We believe, however, that the evidence is sufficiently convincing to enable us to say that from 1·5 to 2 g. of hexadecane or some other saturated substance derived from it has been stored in the tissues other than the liver as a result of feeding. Thus of 149 g. of hexadecane administered, about 114 g. has been excreted in the faeces, and of the remaining 35 g., about 1·85 g. has appeared in the tissues, leaving about 33 g. unaccounted for.

Discussion.

The degree to which the hexadecane has been absorbed needs to be assessed with caution from these results. In exps. I and II the amount of hydrocarbon not recovered in the faeces corresponds to 49 and 50 mg. per rat per day respectively and in exp. III to 108 mg. per day. These figures are open to two possible sources of error: firstly that the accuracy of measurement as determined by the intake and excretion method is limited for the reasons discussed in a previous paper [Channon and Collinson, 1928] and secondly it is not impossible that part of the hexadecane may have been attacked by bacteria in the large intestine, since, as is mentioned later, certain moulds at least are able to utilise higher *n*-hydrocarbons as a sole source of carbon. We may perhaps dismiss the latter possibility as one unlikely seriously to impair the results on account of the slowness with which such an action is likely to take place, while the former should not in any case render the results markedly inaccurate. If therefore the results are accepted at their face value, a number of deductions may be made.

(1) That *n*-hexadecane is absorbed from the alimentary tract of the rat to the extent of between 50 and 100 mg. per day.

This result is essentially similar to those previously obtained with squalene [Channon, 1926] and with a liquid paraffin which was proved to be a mixture of polycyclic hydrocarbons [Channon and Collinson, 1929] in that absorption is definite although the degree of absorption is very limited. It is to be noted that previous experiments suggest that squalene is absorbed even to a less extent than is hexadecane. So far as appears at present therefore the chemical nature of the hydrocarbon has little effect on its degree of absorption which is possibly conditioned by its solubility in bile salt solutions of fatty acids.

(2) In exp. III, in which the unsaponifiable matter from the entire bodies of the animals was prepared, of 35 g. of hexadecane not recovered in the faeces, only about 1·5 g. were present in the tissues, and hence 13 rats have apparently metabolised 33·5 g. in 25 days. Such a finding that the animal appears to possess the power to metabolise a normal aliphatic hydrocarbon is an unexpected one. On the other hand the work of Tausson [1925] and Hopkins and Chibnall [1932] has shown that certain moulds can utilise higher paraffins as a sole source of carbon, while Tausz and Peter [1929] showed that certain soil bacteria possess similar powers. The utilisation of higher normal hydrocarbons by moulds shows that these substances, even though possessing no polar group, are not immune from attack at least by this form of living matter, and hence the disappearance

of some 33 g. of hexadecane in our experiments on the rat may be less surprising than appears at first sight.

(3) The third point worthy of note is that the absorption of hydrocarbon has not resulted in an increase in the percentage of the unsaponifiable matter in the liver. This result is the same as was obtained by Channon and Collinson [1929] when they administered a liquid paraffin to rats, where the percentage of unsaponifiable matter did not rise although the iodine value of the non-sterol fraction was modified in a similar way to that in exp. III of this paper. It was this finding which led these workers to continue the work on pigs from the liver of which the hydrocarbon was isolated. On the other hand, when squalene was administered to rats Channon [1926], the liver unsaponifiable matter rose to about 1 %. It is difficult to suggest why the effects of the administration of squalene which is apparently less well absorbed should cause such a marked rise in the liver unsaponifiable matter, when that of hexadecane causes no rise whatever. Similarly, of the alcohols previously investigated in this experiment phytol, oleyl and cetyl alcohols and cholesterol only the last caused a significant rise in the liver unsaponifiable matter.

(4) The failure of the hydrocarbon to raise the liver unsaponifiable matter significantly is the more surprising in view of the presence of the hydrocarbon in other tissues of the animals, presumably the fat of adipose tissues. During recent years work on the fat-soluble vitamins and the investigations which have been carried out on other substances of peculiar biochemical interest which occur in various fish-liver oils (batyl, selachyl, chimyl and other alcohols, squalene and other hydrocarbons) has tended to focus attention on the liver as the tissue where unsaponifiable substances are generally to be found. This is in part because other tissues, so far as the slight extent to which they have been investigated shows, contain unsaponifiable substances in relatively insignificant amount only. Further, cholesterol is the main constituent of this fraction. Hence it may appear surprising that the hydrocarbon should not be obviously present in the liver. On the other hand, presumably the capacity of the liver for unsaponifiable substances generally must be limited by their nature and therefore it would perhaps be that such a saturated substance as a hydrocarbon would find its way into the fat depots, if it were not readily oxidised.

Summary.

1. Synthetic *n*-hexadecane has been administered to two groups of 9 and 10 rats for periods of 21 days respectively and the degree of absorption measured by the preparation and analysis of the unsaponifiable fractions of the faeces of these and the control groups of animals.

2. The results indicate an absorption by the rat of 50 mg. of hexadecane daily. This absorption had no effect on the amount of the unsaponifiable matter of the livers, nor on their cholesterol content, nor on the iodine value of the non-sterol fraction.

3. In a third experiment 149 g. of synthetic *n*-hexadecane were administered to 13 rats for 25 days, being incorporated in the diet to the extent of 5 %.

4. The degree of absorption measured by the intake and excretion method corresponded to each rat having absorbed 108 mg. per day and resulted in the iodine value of the non-sterol fraction of the unsaponifiable matter of the livers falling from the control value of 122 to 66.

5. After removal of the livers and alimentary tracts, the carcases of the animals were examined for the hydrocarbon, and of the 35 g. of the latter not

recovered from the faeces, evidence was obtained of the presence of 1·85 g. in the tissues.

6. The disappearance of the remaining 33·15 g. suggests that the animal organism can metabolise normal aliphatic hydrocarbons.

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CCII. ON THE THERMOSTABILITY OF PROLAN.

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Many authors have confirmed Zondek and Aschheim's [1928; 1929] original experiments on the thermolability of the ovary-stimulating substance found by them in the anterior pituitary lobe and in the urine of pregnant women[2]. All the observations, however, appear to have been made on material heated in aqueous solution, and the possibility that heating the substance in the dry state might give different results has been overlooked. In attempting high vacuum distillation of the active substance from a dried crude extract of urine, we were led to examine the activity of residues subjected to temperatures of 80°, 100° and 150° for an hour in a high vacuum. It has been found that the active substance of urine of pregnancy is not readily destroyed at 100° when heated in the form of a dry powder, although we have confirmed the fact that heating in the presence of water results in complete and rapid destruction.

Methods and material.

Prolan extract. The same urine extract was used throughout the experiments; it was stored at − 2° as a dry powder, under which conditions it remains indefinitely stable. The extract was crude; 10 mg. caused ovulation in 39 out of 40, and 5 mg. in 33 out of 65 oestrous rabbits [Parkes and White, 1933]. 10 mg. have therefore been considered as the amount required to cause ovulation with reasonable certainty. The heated samples were tested in the same way, by intravenous injection followed next day by a sterile laparotomy under ether to ascertain if ovulation had occurred.

Heating in dry condition. The samples heated *in vacuo* were spread on the inside of a thin-walled glass vessel, which was then evacuated by a two-stage mercury vapour pump connected through a trap cooled with liquid oxygen. The vacuum attained was higher, probably much higher, than 10^{-3} mm. Hg. The evacuated vessel was heated in a suitable liquid bath.

Samples heated in oxygen or nitrogen were placed in an ampoule, which was then alternately evacuated and filled four times with the required gas dried by passage over P_2O_5. The ampoule was sealed off after the final admission of dry gas.

Heating in aqueous solution. For obtaining an oxygen-free solution, the sample was weighed into an ampoule from which air was displaced by a current of nitrogen. 10 cc. of distilled water, boiled and cooled in a current of nitrogen,

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[2] To material from this latter source Zondek and Aschheim have given the name "prolan," which is used here for lack of a better one.

were added. During the heating a slow stream of nitrogen was passed through the solution.

In the converse experiments, oxygen was bubbled through the solution during the heating.

Effect of heating in aqueous solution.

Confirmatory experiments on the thermolability of prolan tested in aqueous solution were carried out as shown in Table I. The results show that:

(*a*) Very brief heating to 100° in water causes destruction. Our actual experimental figures show a loss of not less than 90 % after 5 minutes. Tests on material heated for 1 hour were carried rather further and indicate a loss of not less than 98 %. Destruction is probably complete in both cases.

Table I. *Effect of heating prolan in aqueous solution.*

No. of exp.	Temperature (°)	Duration of heating (hrs.)	Atmosphere	Amount tested (mg.)	Result
3 *a*	100	1	Oxygen	25	−
1 *c*	100	1	Nitrogen	30	−
1 *d*	100	1	Nitrogen	30	−
3 *c*	100	1	Oxygen	100	−
3 *d*	100	1	Nitrogen	500	−
6 *c*	100	$\frac{1}{4}$	Nitrogen	100	−
7 *b*	100	$\frac{1}{4}$	Nitrogen	250	−
7 *a*	100	$\frac{1}{12}$	Nitrogen	100	−
9 *a*	60	1	Nitrogen	10	+
9 *b*	60	1	Nitrogen	10	−
10 *a*	60	1	Nitrogen	10	+
10 *b*	60	1	Nitrogen	10	−
8 *a*	60	1	Air	20	+
8 *b*	60	1	Air	20	+
7 *c*	60	1	Nitrogen	50	−
8 *c*	60	1	Air	50	+
8 *d*	60	1	Air	50	+
6 *a*	60	1	Nitrogen	100	+
8 *f*	60	1	Air	100	+

(*b*) The destructive action does not appear to be influenced by the atmosphere in which the solution is heated.

(*c*) Heating to 60° in water for 1 hour is less destructive. At least 50 % of the activity survives this treatment.

Table II. *Effect of heating prolan in the dry state.*

No. of exp.	Temperature (°)	Duration of heating (hrs.)	Atmosphere	Amount tested (mg.)	Result
GS 7 R	80	$\frac{1}{2}$	Vac.	20	+
GS 9 R	100	1	Vac.	20	+
GS 9 R	100	1	Vac.	10	+
1 *a*	100	1	Nitrogen	10	+
2 *b*	100	1	Oxygen	10	+
2 *c*	100	1	Oxygen	10	−
2 *d*	100	1	Oxygen	10	+
4 *b*	100	1	Oxygen	10	+
4 *c*	100	1	Oxygen	10	+
4 *d*	100	1	Oxygen	10	+
4 *e*	100	1	Oxygen	10	+
GS 11 R	100	1	Vac.	5	−
GS 11 R	100	3	Vac.	10	+
GS 12 R	100	3	Vac.	10	+
6 *b*	150	1	Nitrogen	100	+
8 *e*	150	1	Nitrogen	50	+
GS 13 R	150	1	Vac.	20	−

Effect of heating in the dry condition.

The results obtained by heating the dry powder were in striking contrast to those given by heating aqueous solutions. The experiments are summarised in Table II which shows that 10 mg. of the dry powder heated to 100° for 1 hour or more gave a positive response in 10 out of 11 experiments. Comparing this response with the known activity of the unheated powder, it is evident that resistance to the treatment must have been almost if not quite complete. Even heating to 150° for 1 hour failed to destroy all activity, five times the usual dose being active after such treatment.

Discussion.

The experiments described above demonstrate conclusively that whereas the ovulation-producing substance of urine of pregnancy is readily destroyed by heat in the presence of water, it is remarkably thermostable in the dry state. This difference in stability to heat confirms the suggestion that the destructive action is hydrolytic in nature [Marshall, 1932] but, unfortunately, it is not easy to see what use can be made of the property in the purification of extracts.

On the other hand, one important practical use can be made of the findings recorded above, *i.e.* in sterilising the extract. Adequate sterilisation for injection has hitherto been a serious difficulty in the administration of urine extracts. Bacterial filters suffer from two drawbacks: (*a*) the active principle is absorbed on the asbestos unless the p_H is carefully adjusted, and (*b*) the final product is necessarily a solution, in which form the substance is not stable. By taking advantage of the thermostability in the dry state, it will be easy to enclose within one ampoule the required amount of sterile powder and sterile water, with a suitable arrangement for mixing when required for injection[1].

Summary.

(1) The ovulation-producing substance of urine of pregnancy is readily destroyed by heating in aqueous solution.

(2) The substance is, however, remarkably thermostable in the dry state; exposure to 100° for 1 hour or more causes no detectable loss of activity.

(3) The exclusion of oxygen does not appear to affect the stability either in aqueous solution or the dry state.

[1] Ampoules of this type are already used by pharmaceutical firms for other substances.

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CCIII. THE VITAMINS PRESENT IN OX-SERUM. I.

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(*Received August 9th, 1933.*)

In the investigation described below the presence of vitamins A and B in ox-serum was sought for by means of biological tests.

Sherman and Boynton [1925] have already shown that rat-blood contains vitamin A.

Its presence in serum is a matter of inference, rather than of direct evidence, from the experiments of Euler and Virgow [1932], who showed that the carotene isolated from serum possesses vitamin A activity.

Vitamin B_1 was found absent from blood [Kollath, 1930] while vitamin B_2 was found to be present [Hoagland, 1923].

Technique.

The experiments were carried out with white rats, all being pure bred Wistar stock and reared at the laboratory. The young were put on to the experimental diets when 3–4 weeks old and weighing between 30 and 40 g.

Four diets were used in the experiments having the following composition: the quantities given are the daily rations per rat in g. (or cc. in the case of liquids).

	Diet 1	Diet 2	Diet 3	Diet 4
Caseinogen	3	$2\frac{1}{4}$	3	$2\frac{1}{4}$
Hardened cottonseed oil	2	2	2	2
Rice starch	10	10	10	10
Lemon juice	1	1	1	1
Salt mixture	1	1	1	1
Dried yeast	1	1	1	0
Irradiated cholesterol	1/200	1/200	1/200	1/200
Cod-liver oil	1/20	0	0	1/20
Ox-serum	0	10	0	10
Vitamin content of diet	ABCD	BCD +serum-vitamins	BCD	ACD +serum-vitamins

The salt mixture was based on a formula by McCollum and Davis and suggested by Pryde [1928]. It has the following composition:

	%
NaCl	4·7
$MgSO_4, 7H_2O$	7·1
NaH_2PO_4, H_2O	9·4
K_2HPO_4	25·8
$CaH_4(PO_4), H_2O$	14·6
Ca lactate	35·2
Fe citrate	3·2
KI	Trace (0·003 %)
	100

The cholesterol used was activated by dissolving 2 g. in 100 cc. warm light petroleum and exposing to rays from a mercury vapour quartz lamp at a distance of 12 ins. for 30 minutes. 40 cc. liquid paraffin were then added, and the ether

Table I.

No. of rat	Diet	Vitamins in diet	Initial weight	Gain in weight	Period of observation (days)	Fate of rat
22	1	A, B, C, D	41	159	56	Survived, healthy
23	1	,,	43	142	56	,,
24	1	,,	43	172	56	,,
47	2	B, C, D and serum	29	105	55	,,
48	2	,,	32	136	55	,,
49	2	,,	30	141	55	,,
57	2	,,	38	147	56	,,
58	2	,,	36	109	56	,,
59	2	,,	36	179	56	,,
60	3	B, C, D	39	39	52	Ataxic
61	3	,,	32	28	57	Died
62	3	,,	37	51	54	,,
50	4	A, C, D and serum	32	—	24	,,
51	4	,,	32	3	25	,,
52	4	,,	33	5	25	,,

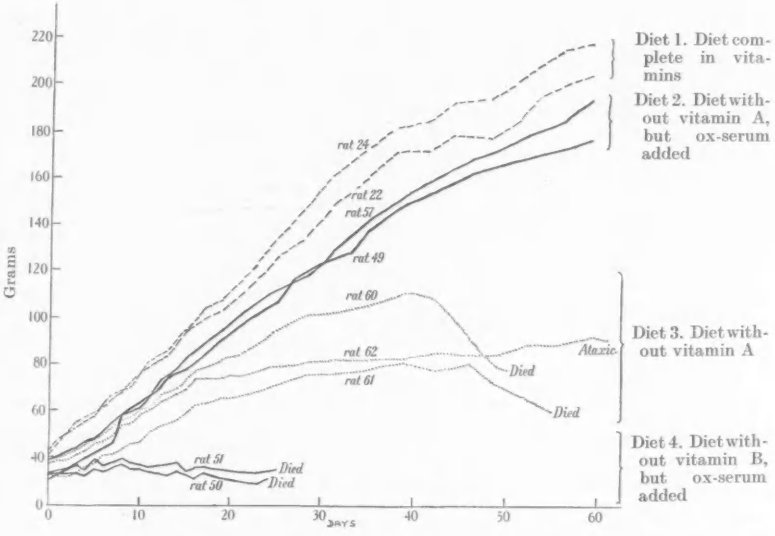

Fig. 1. Chart showing that serum contains vitamin A but not the vitamin B complex.

was evaporated off on a steam-bath. The resulting solution contained 5 mg. irradiated cholesterol in 0·1 cc. oil. This amount was administered daily to the rats by means of a graduated pipette.

In the case of rats receiving cod-liver oil in addition to irradiated cholesterol, a mixture was prepared, 0·1 cc. of which contained 5 mg. of cholesterol and

0·05 cc. cod-liver oil. This was obtained by adding 20 cc. liquid paraffin and 20 cc. cod-liver oil to the irradiated solution of 2 g. cholesterol in 100 cc. light petroleum and evaporating off the ether.

The diets were so prepared that the addition of serum did not increase the calorific value of the diet. Thus when a daily ration of 10 cc. serum was allowed, the corresponding amount of protein was deducted from the caseinogen ration. As the serum used had a protein content of 7·5 % (as determined by the Kjeldahl method), a 10 cc. ration represented 0·75 g. protein: rats receiving serum were therefore given 2·25 g. caseinogen instead of 3 g. daily.

In the latter part of the experiment lemon juice was not added to the diets. This omission, however, was not made until it had been definitely established by other experiments that lack of vitamin C did not affect the rats in weight or in any other way over a period of several months longer than the duration of the present experiment (two months).

15 rats were used in the investigation: 3 were put on diet 1 (complete in vitamins); 6 on diet 2 (containing serum instead of vitamin A); 3 on diet 3 (containing no vitamin A); 3 on diet 4 (containing serum instead of vitamin B). The rats were weighed at least twice a week.

The results are indicated in Table I and Fig. 1.

Results.

Vitamin A. From Table I and the accompanying chart it is clear that serum contains vitamin A, since when serum is added to a diet deficient in vitamin A growth proceeds normally.

Since the average rate of growth of the rats was 2·8 g. per day on diet 1 and 2·5 g. per day on diet 2, the vitamin A content of 10 cc. serum is approximately 90 % of that of 0·05 cc. cod-liver oil; *i.e.* the vitamin potency of the serum used was 1/200 (0·45 %) that of the sample of cod-liver oil used.

Vitamin B. As the animals on diet 4 died in 3–4 weeks with scarcely any gain in weight, it is clear that the vitamin B complex is not present in serum. The possibility exists, however, of one or other of the components of vitamin B being present in serum.

Conclusion.

Ox-serum contains vitamin A but does not contain the vitamin B complex.

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CCIV. THE ACTION OF VITAMIN C ON THE OXIDATION OF TISSUES *IN VITRO*.

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A few years ago, Szent-Györgyi [1928] isolated from the adrenal cortex a strongly reducing substance, "hexuronic acid." He showed that this substance could be reversibly oxidised and reduced, and suggested that it might play the part of an intermediary hydrogen carrier in tissue respiration. Recently, Svirbely and Szent-Györgyi [1932] produced strong evidence that "hexuronic acid"—since renamed ascorbic acid—is identical with vitamin C, and subsequent work has largely confirmed this.

It seemed probable that if vitamin C does play a part in tissue respiration, it might be possible to demonstrate its action by adding ascorbic acid to tissues which had been depleted of their store of vitamin C, that is, to the tissues of animals suffering from scurvy, and testing whether the addition of the vitamin increased the surviving respiration of the tissue. On carrying out the experiment, using the tissues of scorbutic guinea-pigs, it was found that the addition of vitamin C even in very small quantities does bring about a definite increase in the respiration of the tissues *in vitro*.

Experimental.

Guinea-pigs weighing about 300 g. were fed on a scurvy-producing diet consisting of bran, 60 %; rolled oats, 30 %; separated milk powder (heated for 2 hours in the oven at 120° with occasional stirring), 10 %. Water was given *ad lib.* and each animal received 0·5 cc. cod-liver oil weekly. The normal (non-scorbutic) control animals received the above diet with the addition of 3 to 5 cc. of fresh lemon juice daily. On this diet, the animals without lemon juice began to lose weight in about 3 weeks and showed definite symptoms of scurvy, while the control animals continued to grow. The animals were usually taken for experiment after about 3 weeks on the diet, though some were taken earlier than this. Equal numbers of male and female animals were used. The tissues examined were liver and muscle.

The guinea-pig was killed by a blow on the neck and allowed to bleed, and the tissues were quickly taken out and prepared, the liver by cutting thin slices by hand with a razor, and the leg muscle by cutting into small pieces with sharp scissors. Samples of the tissue were then weighed out on the torsion balance and suspended in phosphate buffer at p_H 7·4, either with or without vitamin C, in the bottles of the Barcroft differential manometric apparatus. Phosphate buffer was used in the experiments rather than a bicarbonate-Ringer solution, since it was desired to follow the course of the reaction, and in the manometric methods involving the use of bicarbonate buffer in equilibrium with a CO_2 gas mixture, it is only possible to take one final reading of oxygen uptake. The phosphate buffer

was prepared by treating $M/5$ KH_2PO_4 solution with the requisite amount of $N/5$ NaOH and diluting to 4 times its original volume. In the earlier experiments, the liver slices were cut with a razor moistened with buffer solution and were then washed in phosphate buffer, blotted on filter-paper and weighed. It was found, however, that much better agreement between duplicates was obtained if the liver slices were cut with a razor moistened only by the liver tissue fluid itself, the unwashed slices then being well mixed on a watch glass, divided into approximately equal portions with sharp scissors and equal amounts weighed out on the torsion balance. This technique was used in the majority of the experiments described in this paper, and the agreement between duplicate experiments on the same tissue was usually within 5 %.

In parenthesis, it may be mentioned that a very convenient method of checking the constants of the manometric apparatus was found to be given by the oxidation of alkaline ferrous sulphate. A suitable amount (depending on the range of the apparatus) of standard ferrous sulphate solution, slightly acidified with H_2SO_4, was run into each right-hand bottle from a micro-burette, and, after bringing the apparatus into equilibrium in the bath and closing the taps, excess of NaOH was tipped into the solution. This can readily be done from a small tube suspended on the soda tube of the bottle. The oxygen uptake due to the oxidation of the ferrous iron reaches completion in about 15 minutes, and the method has the advantage that the calibration can be done under the same conditions as those under which the actual experiments are carried out.

The manometers used for the controls without vitamin C and the experiments with vitamin C were frequently interchanged.

Two different samples of ascorbic acid were used in these experiments. Both were colourless, crystalline preparations and, when titrated with iodine, took up exactly 2 atoms of iodine per molecule. The ascorbic acid solution was prepared freshly just before each experiment by dissolving the crystalline acid in a small volume of phosphate buffer and bringing the p_H back to 7·4 by adding the theoretical amount of $N/5$ NaOH. An equal amount of ascorbic acid in phosphate buffer but without tissue was always added to the left-hand bottle of the apparatus to compensate for the small oxygen uptake due to the autoxidation of ascorbic acid itself. In all experiments, the total volume of fluid in each bottle was 3·0 cc. The bottles were filled with oxygen rather than with air in order to reduce the possibility that the thickness of the tissue might be a limiting factor in the rate of diffusion and uptake of oxygen. A further reason for using oxygen was that the properties of ascorbic acid suggested that it might function as an intermediary hydrogen carrier between the reducing systems of the cell and activated oxygen. If this were so, its catalytic effect might be expected to be greater if the oxygen-activating systems of the cell were saturated with oxygen.

In filling with oxygen, the technique of Dixon and Tunnicliffe [1923] was used, the vessels being evacuated simultaneously to a pressure of about 100 mm. Hg and allowed to fill with oxygen, the evacuation and filling being carried out three times. The manometers were then shaken in a water-bath at 37°, allowing them to shake in the bath for 10 minutes before closing the taps and commencing to take readings. The CO_2 given off by the tissue was absorbed by rolls of starch-free filter-paper soaked in 40 % KOH in the small inset tubes in the bottles, and the oxygen uptake was measured over a period of 2 hours. About 45 minutes usually elapsed between the killing of the animal and the first reading on the manometer. Most of the determinations were carried out in duplicate on the same tissue both with and without vitamin C, and the mean values of oxygen uptake of the pairs with and without the vitamin respectively were compared.

Table I shows the effect of 0·25 mg. of ascorbic acid (concentration 0·08 mg. per cc.) on the oxygen uptake of liver slices from scorbutic guinea-pigs. The oxygen uptake figures are the totals over a period of 2 hours, and the tissue weights are given as wet weights. All the experiments, with the exception of the first three in the Table, were carried out in duplicate, both with and without addition of the vitamin, a total of 17 scorbutic guinea-pigs being used.

Table I. *Oxygen uptake of scorbutic liver respectively with and without 0·25 mg. vitamin C.*

Tissue weight in mg.	Alone mm.^{3} O_2	+Vitamin C mm.^{3} O_2	Difference	Percentage increase
154	194	232	38	+20
174	252	272	20	+ 8
237	130	205	75	+57
300	466	541	75	+16
300	527	566	39	+ 8
285	286	370	84	+29
300	400	503	103	+26
225	144	164	20	+14
300	361	547	186	+52
185	145	173	28	+19
100	158	183	25	+16
255	523	600	77	+15
250	357	404	47	+13
152	338	373	35	+10
300	437	458	21	+ 5
207	405	450	45	+11
200	230	349	119	+52

It can be seen that there is a variable but very definite increase in the oxygen uptake of scorbutic guinea-pig liver produced by the addition of 0·08 mg. per cc. of vitamin C.

A few experiments have been carried out with 0·1 mg. ascorbic acid in 3 cc. and a definite increase in the oxygen uptake of scorbutic liver was produced. In one experiment with 0·05 mg. of the vitamin in 3 cc., a completely negative result was obtained.

Table II shows the effect of addition of 0·25 mg. of ascorbic acid in 3 cc. on the oxygen uptake of liver slices from nine normal guinea-pigs. Apart from the first two experiments in the table, the experiments were all carried out in duplicate and the conditions were identical with those in the experiments with scorbutic liver.

Table II. *Oxygen uptake of normal liver with and without 0·25 mg. vitamin C.*

Tissue weight in mg.	Alone mm.^{3} O_2	+Vitamin C mm.^{3} O_2	Difference	Percentage difference
300	793	762	−31	− 4
215	625	619	− 6	− 1
*197	91	95	+ 4	+ 4
214	416	367	−49	−12
200	467	466	− 1	0
232	400	384	−16	− 4
200	328	328	0	0
300	720	659	−61	− 8
283	464	459	− 5	− 1

* In this experiment, the tissue had stood in air for 2 hours before testing.

It is clear that no increase is produced by addition of the vitamin to liver slices from normal guinea-pigs, in fact, if anything, there is a slight decrease in the oxidation in presence of ascorbic acid.

It should be mentioned that the results both in this table and those with scorbutic animals in Table I are in no way selected. They are the results of all the experiments I have carried out with the exception of one which was carried out on a guinea-pig obtained from another laboratory and believed to be suffering from scurvy. In this case, no increase in oxygen uptake was obtained on adding 0·25 mg. ascorbic acid to the liver slices, but on *post mortem* examination no signs of scurvy could be found in the animal.

It was not found possible to correlate the amount of increase in oxygen uptake produced on addition of the vitamin with the degree of scurvy from which the animal was suffering. While most of the scorbutic guinea-pigs showed definite haemorrhages around the knee joint, some of the biggest increases in oxygen uptake were obtained with animals which had been fed for a relatively short time on the scorbutic diet and which, though showing symptoms of scurvy on *post mortem* examination, appeared lively and well nourished. It appears very unlikely, therefore, that the effects observed were due to the inanition which occurs in the later stages of scurvy.

It was observed that in most of the experiments with scorbutic tissue, the accelerating effect of vitamin C on the oxygen uptake became much greater during the second hour than it was during the first hour, and the percentage increase in uptake produced by the vitamin calculated on the second hour alone gave much larger figures than those shown in Table I. Possibly this may be due to the slow diffusion of the vitamin into the tissue, or to a small residual quantity of vitamin C in the scorbutic tissues which becomes used up, the effect of added vitamin C then becoming greater.

No attempt has been made to calculate the results in terms of oxygen uptake per g. of tissue, since it was found consistently that under the conditions of these experiments, the oxygen uptake per g. of tissue is dependent on the amount of tissue taken. Using two different weights of the same tissue, the uptake per g. was always greater with the greater weight of tissue. Possibly some oxidative catalyst diffuses out of the cell into the buffer, and the velocity of oxygen uptake of the tissue depends on the concentration of this substance in the surrounding fluid. It is consequently difficult from the results in Tables I and II to calculate average figures for the oxygen uptake per g. of scorbutic and normal tissues. A further difficulty is that the time elapsing between the death of the animal and the first measurement varied in different experiments. On the whole, however, in comparing experiments in which equal or approximately equal weights of liver were used, it appears that the oxygen uptake of the scorbutic tissue is definitely lower than that of the normal tissue. The addition of ascorbic acid tends to raise the uptake of the scorbutic tissue towards that of normal tissue rather than to increase the uptake above the normal value.

A few experiments have been carried out on muscle, but these experiments have been less satisfactory, since I have been unable to prepare thin slices of muscle, and when chopped muscle is used a great part of the oxygen uptake occurs while the apparatus is being brought into equilibrium in the thermostat. The experiments indicate, however, that both with 0·1 mg. and 0·25 mg. of ascorbic acid, there is a slight increase in the oxidation of the muscle of normal animals and a relatively large increase in the oxidation of muscle taken from scorbutic animals, though too few experiments have been done to justify a quantitative comparison.

Recent experiments by Harris and Ray [1933] indicate that the guinea-pig requires about one mg. a day of vitamin C for normal maintenance. It seems therefore that in the experiments that I have described, in which the effects in oxidation have been produced *in vitro* by the vitamin in concentrations of 0·08 mg. per cc. or less, the vitamin is acting in concentrations which are not unphysiologically large.

That these findings have some relation to events in the intact animal is indicated by a recent paper by Söderström and Törnblom [1933], in which it is shown that feeding guinea-pigs on a scurvy-producing diet results in a marked decrease in the uptake of oxygen by the intact animals.

It is probably premature to attempt to speculate on the relation of these findings to scurvy as a disease. It is not difficult to imagine, however, that impeded oxidation in the scorbutic animal might lead to loss of tone in the capillary muscles, leading to increased permeability and the characteristic haemorrhages associated with scurvy.

Tissues other than liver and muscle have not so far been examined. Gavrilescu *et al.* [1932] have shown that the action of vitamin B_1 concentrates in restoring the lowered oxygen uptake of polyneuritic pigeon's brain is specifically concerned with the glucose-lactate enzyme systems. Experiments designed to determine what oxidising system or systems are linked up with the activity of vitamin C are now in progress.

SUMMARY.

The surviving respiration of tissues taken from normal and scorbutic guinea-pigs was measured manometrically in oxygen, both with and without addition of vitamin C (ascorbic acid). With scorbutic animals, using thin slices of liver suspended in phosphate buffer (p_H 7·4), the oxygen uptake is lower than that of liver from normal animals.

The addition of 0·25 mg. of ascorbic acid in 3 cc. brings about an increase in the oxygen uptake of scorbutic liver slices amounting to 5 to 57 % (average 25 %). With normal guinea-pigs, the oxygen uptake of the liver was unaffected or even slightly decreased by the addition of 0·25 mg. of ascorbic acid. Using chopped muscle, the oxygen uptake with normal guinea-pigs was slightly increased with 0·1 to 0·25 mg. ascorbic acid, while with muscle from guinea-pigs suffering from scurvy, a relatively large increase in oxygen uptake was obtained.

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CCV. THE EFFECT OF TESTICULAR HORMONE ON NORMAL SEXUALLY MATURE RATS. A METHOD OF BIOLOGICAL ASSAY.

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From the Lister Institute, London.

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In the previous paper [Korenchevsky *et al.*, 1933, 1] a study was made of the influence of a purified preparation of testicular hormone isolated from human urine on young normal rats, about one month old, *i.e.* on rats sexually immature at the beginning of the injections. The injected animals, as compared with the controls, showed depression of the development of the testes in most cases with small doses and in all rats with large doses. The prostate with seminal vesicles and the penis were, in most cases, slightly decreased in weight with small doses, whilst with large doses an increase in weight was obtained and also an acceleration in the involution of the thymus and a slight increase in the growth of the kidney. No large or constant changes were found in the weights of the other organs or in the body weight. In the present paper a description is given of experiments performed on normal adult rats which had reached sexual maturity at the beginning of the injections.

Technique.

Experiments were performed on 37 rats belonging to 9 litters. The ages and weights of the rats are given in Table I. The age at the beginning of the experiment of the first six litters of 29 rats was from 74 to 77 days. The 4 rats of

Table I. *Age and average weight of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Initial age (days)	Final weight of rats			
			Controls	0·5 R.U.	1 R.U.	10 R.U.
1	6	77	330	313	—	329
2	5	75	305	290	—	312
3	3	75	322	—	325	—
4	5	75	361	—	380	—
5	5	75	312	—	350	297
6	5	74	364	—	—	341
7	4	134	341	—	—	344
8	2	261	476	—	—	401
9	2	About 1 year	595	—	—	456

litter 7 were used in the present experiment about 3 weeks after the end of another experiment in which they had been fed on a diet partially deficient in vitamin D. During the intervening three weeks they were fed on a complete diet with the addition of 5 drops of cod-liver oil daily. For this reason the results with this litter, as well as those with the four much older rats of litters 8 and 9, are not included in the averages in Tables III to VI, but are given separately in these

Tables. The results of litters 8 and 9 have been averaged since each litter consisted of only two rats, one control and one injected rat.

A preparation of testicular hormone, isolated from human urine and dissolved in sesame oil, prepared by Messrs Schering Ltd., was used for the injections[1]. This preparation was assayed by us, using our method of assay [Korenchevsky *et al.*, 1932] and was found to contain about 50 rat units per cc. In the experiments two small doses of 0·5 R.U. and 1 R.U. and one large dose of 10 R.U. per day were used, injected in half doses twice a day, the period of injection being 21 days in litters 1 to 7 and 10 days in litters 8 and 9. The rest of the technique was the same as that used by us in our previous experiments [Korenchevsky *et al.*, 1933, 1].

Influence on the weight of the rats.

As can be seen from Tables I and II, neither the difference in the final weight nor in the gain in weight was of such a degree or constancy that any definite significance could be attributed to these with the possible exception of the rats

Table II. *Influence on gain in weight.*

Average gain in weight (g.) in each group of each litter after the injection of testicular hormone.

	Actual gain				Gain per 100 g. of initial weight			
		Rats injected with				Rats injected with		
No. of litter	Control rats	0·5 R.U.	1 R.U.	10 R.U.	Control	0·5 R.U.	1 R.U.	10 R.U.
1	55	42	—	56	20	15	—	21
2	43	44	—	48	16	18	—	18
3	40	—	47	—	14	—	17	—
4	49	—	67	—	16	—	21	—
5	22	—	55	25	8	—	19	9
6	53	—	—	43	17	—	—	14
7	35	—	—	31	11	—	—	10
8+9	9	—	—	10	2	—	—	4

injected with 1 R.U. All the rats of this latter group in all three litters gained more than either their controls or the rats of the same litter injected with other doses of the hormone. In litters 3, 4 and 5 the average gain in weight per 100 g. of initial weight of rats was 21·4 %, 31·3 % and 137·5 % more than that of the controls.

Influence on testes.

The results are summarised in Table III. The changes in weight of the testes were small, more particularly when considering the results calculated for the same unit of body weight, being on the average in the three different groups of litters 1 to 6, − 2·7 %, + 5·3 % and − 9·2 % as compared with the average for the control group.

In litters 7 to 9, of older rats, a decrease was noted in the weight of the testes, when calculated per 200 g. of body weight, of 21·1 % and 27·4 %. As can be seen from Table III, a constant although small decrease in the weight of these glands was only obtained with the large dose of 10 R.U., the decrease occurring in all litters except litter 5. A preliminary histological examination of the testes of the injected rats in no case revealed any changes in the seminiferous cells or in spermatogenesis. The Leydig's cells in the testes of rats injected with the large dose of 10 R.U., however, seemed to contain less protoplasm and had smaller nuclei than those of either the control rats or of those injected with 0·5 and 1 R.U. Detailed histological results will be published elsewhere.

[1] We wish to express our thanks to Messrs Schering Ltd., for kindly supplying this preparation.

Table III. *Influence on testes.*

The average weights (g.), actual and calculated per 200 g. of body weight, of testes of normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with			Percentage change		
		0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.
A. Actual weights.							
1	2·581	2·408	—	2·173	−6·7	—	−15·8
2	2·952	2·677	—	2·511	−9·3	—	−14·9
3	2·228	—	2·575	—	—	+15·6	—
4	3·215	—	3·211	—	—	− 0·1	—
5	2·340	—	2·735	2·292	—	+16·9	− 2·1
6	3·076	—	—	2·649	—	—	−13·9
Average	**2·732**	**2·543**	**2·840**	**2·406**	**−8·0**	**+10·8**	**−11·7**
7	2·022	—	—	1·598	—	—	−21·0
8+9	3·592	—	—	2·111	—	—	−41·2
B. Weights per 200 g. of body weight.							
1	1·565	1·545	—	1·337	−1·3	—	−14·6
2	1·935	1·855	—	1·611	−4·1	—	−16·7
3	1·384	—	1·608	—	—	+16·2	—
4	1·785	—	1·688	—	—	− 5·4	—
5	1·500	—	1·577	1·544	—	+ 5·1	+ 2·9
6	1·693	—	—	1·554	—	—	− 8·2
Average	**1·644**	**1·700**	**1·624**	**1·512**	**−2·7**	**+ 5·3**	**− 9·2**
7	1·191	—	—	0·940	—	—	−21·1
8+9	1·357	—	—	0·985	—	—	−27·4

Thus with the small doses no definite changes in the testes were obtained, while after the injection of the large dose a slightly depressing effect on the Leydig's cells and in most litters a small decrease, if any, in the weight of the testes were indicated.

Influence on prostate with seminal vesicles and penis.

No definite effect on the weight of these sexual organs (Tables IV and V) was found as the result of the injection of testicular hormone, the variations obtained being small and not constant.

However, it is significant that in most litters a decrease of prostate with seminal vesicles, although slight, was present, indicating that the preparation had no stimulating effect on these organs in adult normal rats.

Influence on thymus.

In all the rats receiving injections of 1 and 10 R.U. the thymus was smaller than in their controls, the average decrease in weight, calculated per 200 g. of body weight, being 23 % and 25·5 % respectively for the six litters of younger rats, while the decrease in litter 7 was 50·4 % and in litters 8 and 9 was 18·6 % (Table V).

Influence on kidneys.

In the previous paper [Korenchevsky *et al.*, 1933, 1] the injection of testicular hormone into young normal rats was shown to be followed by a slight increase in the weight of the kidneys.

In the present experiment this increase was only found consistently in those rats of the first six litters which were injected with 10 R.U. the average increase

Table IV. *Influence on prostate with seminal vesicles.*

The average weights (g.), actual and calculated per 200 g. of body weight, of the prostate and seminal vesicles of the normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with			Percentage change		
		0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.
A. Actual weights.							
1	1·685	1·489	—	1·880	−11·6	—	+11·6
2	1·823	1·298	—	2·088	−28·8	—	+14·5
3	1·482	—	1·476	—	—	− 0·4	—
4	2·173	—	2·041	—	—	− 6·1	—
5	1·859	—	1·744	1·764	—	− 6·2	− 5·1
6	2·386	—	—	1·578	—	—	−33·9
Average	**1·901**	**1·394**	**1·754**	**1·828**	**−20·2**	**− 4·2**	**− 3·2**
7	2·351	—	—	2·458	—	—	+ 4·6
8+9	3·596	—	—	2·665	—	—	−25·9
B. Weights per 200 g. of body weight.							
1	1·022	0·954	—	1·151	− 6·7	—	+12·6
2	1·195	0·896	—	1·340	−25·0	—	+12·1
3	0·920	—	0·910	—	—	− 1·1	—
4	1·205	—	1·072	—	—	−11·0	—
5	1·191	—	0·994	1·188	—	−16·5	− 0·3
6	1·314	—	—	0·927	—	—	−29·5
Average	**1·141**	**0·925**	**0·992**	**1·152**	**−15·9**	**− 9·5**	**− 1·3**
7	1·379	—	—	1·433	—	—	+ 3·9
8+9	1·400	—	—	1·252	—	—	−10·6

Table V. *Influence on penis, thymus, kidneys and intra-abdominal fat.*

Average percentage changes in weight (actual and per 200 g. of body weight) of the organs in each group of each litter of the rats injected with testicular hormone, as compared with those of control rats.

No. of litter	Penis			Thymus			Kidneys			Intra-abdominal fat		
	0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.
A. Actual weights												
1	−14·5	—	− 2·5	+18·4	—	−21·0	+ 0·2	—	+14·4	−5·0	—	+ 2·2
2	−11·8	—	+ 9·0	− 2·0	—	−11·5	−18·6	—	+ 6·9	+2·2	—	+ 1·1
3	—	+ 4·2	—	—	−23·1	—	—	+17·4	—	—	−27·7	—
4	—	− 0·7	—	—	− 6·3	—	—	+ 4·8	—	—	+19·7	—
5	—	− 2·1	− 1·8	—	−29·3	−48·3	—	+18·5	+14·8	—	+70·7	−19·3
6	—	—	−10·5	—	—	−26·6	—	—	+ 4·7	—	—	−31·6
Average	**−13·2**	**+ 0·5**	**− 1·5**	**+ 8·2**	**−19·6**	**−26·9**	**− 9·2**	**+13·6**	**+10·2**	**−1·4**	**+20·9**	**−11·9**
7	—	—	+14·6	—	—	−50·2	—	—	+ 9·4	—	—	+ 8·4
8+9	—	—	+ 2·5	—	—	−31·1	—	—	−22·5	—	—	−16·8
B. Weights per 200 g. of body weight.												
1	− 9·3	—	− 1·6	+24·9	—	−20·1	+ 5·8	—	+15·1	−1·9	—	+ 1·7
2	− 7·1	—	+ 6·7	+ 3·9	—	−13·5	−14·1	—	+ 4·5	+7·3	—	− 1·1
3	—	+ 3·8	—	—	−23·2	—	—	+16·7	—	—	−28·0	—
4	—	− 6·1	—	—	−10·1	—	—	− 0·6	—	—	+13·9	—
5	—	−12·0	+ 3·2	—	−35·6	−45·7	—	+ 4·8	+20·8	—	+48·9	−15·2
6	—	—	− 4·5	—	—	−22·5	—	—	+11·7	—	—	−28·3
Average	**− 8·2**	**− 4·8**	**+ 1·0**	**+14·4**	**−23·0**	**−25·5**	**− 4·2**	**+ 7·0**	**+13·0**	**+2·7**	**+11·6**	**−10·7**
7	—	—	+12·5	—	—	−50·4	—	—	+ 8·7	—	—	+ 7·9
8+9	—	—	+26·9	—	—	−18·6	—	—	− 4·5	—	—	+ 8·4

being 13 %. The changes in the kidneys of the rats injected with the small doses and in those of the old rats were less constant (Table V).

Influence on other organs.

No definite influence could be seen on the fat deposition (Table V) or on the weights of the adrenals, thyroid, hypophysis, liver, spleen or heart (Table VI).

Table VI. *Influence on adrenals, thyroid, hypophysis, liver, spleen and heart.*

Average percentage changes in weight (actual and per 200 g. of body weight) of organs of the injected rats as compared with those of the control rats.

Organs	No. of litter	Actual weights			Weights per 200 g. of body weight		
		0·5 R.U.	1 R.U.	10 R.U.	0·5 R.U.	1 R.U.	10 R.U.
Adrenals	1 to 6	− 9·1	+10·2	− 1·1	−3·9	+ 4·4	+ 1·8
	7	—	—	− 9·2	—	—	−11·9
	8+9	—	—	−19·6	—	—	− 3·4
Thyroid	1 to 6	−12·7	+ 7·1	+ 1·7	−8·0	+ 0·7	+ 4·2
	7	—	—	− 2·8	—	—	− 4·6
	8+9	—	—	− 9·1	—	—	+11·0
Hypophysis	1 to 6	− 8·0	+ 7·7	+ 7·0	−1·7	+ 2·5	+ 9·9
	7	—	—	− 6·7	—	—	− 8·1
	8+9	—	—	− 2·8	—	—	+22·2
Liver	1 to 6	− 5·9	+10·5	+ 3·1	−0·9	+ 6·2	+ 5·3
	7	—	—	+ 4·1	—	—	+ 3·2
	8+9	—	—	−16·5	—	—	+ 3·8
Spleen	1 to 6	− 6·6	+ 6·9	− 2·2	−0·9	+ 1·4	+ 0·5
	7	—	—	± 0	—	—	− 2·1
	8+9	—	—	−24·1	—	—	− 3·7
Heart	1 to 6	− 7·4	+11·4	+ 0·1	−2·4	+ 5·5	+ 2·8
	7	—	—	− 3·3	—	—	− 3·1
	8+9	—	—	−13·2	—	—	+15·3

Discussion.

The change in weight of the organs, used as a method of assay of different substances as applied to normal rats.

In previous papers [Korenchevsky, 1930, 1932, 1, 2] a method was proposed for the accurate estimation of changes in organs as judged by their change in weight, the weighing being made of the organs fixed in Bouin's fixative. The method gives very accurate results provided the other conditions of the method are fulfilled; its accuracy was proved when applying it to the study of the changes produced by castration and cryptorchidism in male rats [Korenchevsky, 1930] and those produced by testicular hormone preparations in castrated and cryptorchid rats (Korenchevsky *et al.*, 1932; 1933, 1, 2, 3].

The data obtained with adult rats, given in Tables III to VI of the present paper, together with the figures obtained on young rats, given in Tables VIII to XII in the previous paper (Korenchevsky *et al.*, 1933, 1, pp. 570–572], show that the method when applied to normal rats also gives results of a satisfactory accuracy. The variations in the average weights in the different groups of the same litter (where the injection was without effect) were small, especially when the weights of the organs were calculated for the same unit of body weight. These variations become smaller, sometimes even insignificant, if the mean of the average weights of the organs of at least three litters is taken. The importance of this latter consideration has been emphasised in our previous papers.

Taking for example the figure for litters 1 to 6 (Table VI of the present paper) it can be seen that of the 18 figures giving the mean variations of the average changes in weight of organs, as compared with that in control rats of 6 litters, none exceeds 9·9 % and in 13 cases they are less than 5 % when calculated per 200 g. of body weight).

The method may, therefore, be considered to be accurate and reliable also with normal animals and might with advantage be applied, not only to the study of various physiological and pathological conditions, but also to the assay of substances having a specific influence on the weight of one or more organs.

On the influence on normal male rats of the preparation of testicular hormone, isolated from urine.

The strength of the testicular hormone used in the present experiments was similar to that of batch I which was injected into young normal rats in the previous experiments [Korenchevsky *et al.*, 1933, 1, pp. 568–573, see litters 6, 7 and 8]. Therefore, although a special assay of batch I was not made, the doses described in the previous paper as 0·25 D, 0·5 D and 2 D were probably about 1·25 R.U., 2·5 R.U. and 10 R.U. respectively. These doses had a depressing effect on the testes of young rats, more particularly, as is shown by the histological examination, on the seminiferous tissue. As judged by changes in the weights of the organs, the small doses of 1·25 R.U. and 2·5 R.U. had also a depressing effect on the prostate with seminal vesicles and penis, while the large dose of 10 R.U. had a stimulating effect.

In the present experiments on adult sexually mature rats only the large dose of 10 R.U. seemed to have any effect of the kind described above, the weight of the testes being slightly decreased in most litters by this dose. Histologically there was a slightly depressing effect on the Leydig's cells but spermatogenesis and the seminiferous tissue seemed normal.

Therefore, injections started before sexual maturity have a definitely depressing effect on the sexual organs, but if started after sexual maturity the effect, if any, is very slight even with large doses.

Comparison of the data of the present experiments with those of the previous experiments [Korenchevsky *et al.*, 1933, 1] shows that the rate of retrogression of the thymus was accelerated both in sexually immature and mature rats, while the slightly stimulating effect of the testicular hormone on the kidneys was not so marked in the mature as in the immature animals.

No definite changes, in either mature or immature animals, were noticed in the weights of the other organs.

Summary.

1. The changes produced by injections of a purified preparation of testicular hormone extracted from urine were studied on 37 normal adult male rats belonging to 9 litters.

2. The physiological variations from the average of the weights of the organs investigated were small.

3. Therefore, by comparing the mean of the average changes in the litters and following exactly the technique and precautions given in the previous papers, the method described could be applied to normal rats for the study of physiological or pathological conditions or for the assay of substances which influence the weight of one or more organs.

4. The effect of injections of the testicular preparation on adult rats was small, as judged by changes in the weight of the organs and the histological examination of the testes, differing in this respect from the quite definite effect

on some of the organs of young rats, since only the large dose of 10 R.U. seemed to have a slightly depressing effect on the weight of the testes and the Leydig's cells.

5. Acceleration in the rate of retrogression of the thymus was noticed in all the rats injected with 1 and 10 R.U.

6. The increase in the weight of the kidneys was very small, being present in most of the groups injected with the large dose.

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CCVI. SIMULTANEOUS ADMINISTRATION OF TESTICULAR HORMONE WITH ANTUITRIN AND PROLAN OR WITH DESICCATED THYROID.

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It is now well known that gonad-stimulating hormones, extracted from the anterior lobe of the hypophysis, or from pregnancy urine, do not influence the secondary sexual organs of castrated animals, since these hormones produce their effects by stimulation of the internal secretion of the testes, or possibly by activation of testicular hormone.

We were unable to find any data in the literature about the action of thyroid hormone on the sexual organs of castrated animals, and as our own investigations on this subject are not yet completed, they will be published later elsewhere. They already show, however, that if there is any influence on the secondary sexual organs of castrated rats it is only slight.

The aim of the present experiments is to investigate whether there is any relationship between the action of testicular hormone, injected into castrated male rats, and that of the thyroid or the anterior lobe of the hypophysis, when introduced simultaneously. By performing these experiments on castrated animals the activity of living testicular cells was excluded. Therefore, any changes obtained differing from the effect of testicular hormone alone should be ascribed to the direct alteration of activity of testicular hormone caused by hormones of the anterior lobe of the hypophysis or of the thyroids.

Since in castrated animals both the latter glands are present, the introduction of an additional amount of the hormones of the anterior lobe or of the thyroid should result in a condition similar to that caused by hypersecretion of these glands.

Technique.

It is unnecessary to discuss here whether the gonad-stimulating substances extracted from urine are completely identical with the hormones of the anterior lobe of the pituitary; in any case their influence on sexual glands is very similar. The preparation from urine was chosen in the present experiments, since such preparations of good quality are much more easily obtained than those extracted from the anterior lobe. We have used "antuitrin S," prepared by Messrs Parke, Davis and Company, for 2 experiments and a mixture of antuitrin S and "prolan," prepared by Messrs Bayer Products Ltd., for 3 experiments (see Table I). These preparations were introduced subcutaneously. Preliminary experiments on young and adult normal male rats have shown that both antuitrin and prolan were very active on the sexual organs in the doses used. As the source of thyroid hormone, desiccated thyroid gland, prepared by Messrs The British Drug Houses Ltd., was given. Each dose of thyroid powder was mixed with about

0·5 g. of wheat germ, moistened with water and given to the rats in small pellets before food; this mixture was greedily consumed in a few minutes. The amount of thyroid fed was calculated according to a special unit of body weight. The daily doses are given in Table I. A testicular hormone preparation, isolated from urine, and prepared and assayed on capons by Messrs Schering Ltd.[1] was used. Different batches of this preparation were used for the various experiments, as it was only supplied in comparatively small quantities at a time. We have not re-assayed the testicular preparations in rat units and in Table I the daily dose is stated in capon units as assayed by Messrs Schering Ltd. Different doses of testicular preparation were given to the rats of different litters (see Tables I and II). The daily doses of testicular and gonad-stimulating hormones were

Table I. *Daily dose of hormones; age and average weight of the castrated rats in each group of each litter.*

	Daily dose of hormones					Age in days		No. of days after castration at beginning of injections	Final weight of rats		
										Receiving hormone	
No. of litter	Testicular c.u.	Antuitrin S r.u.	Antuitrin S + prolan r.u.	Thyroid in mg. per g. of body weight	No. of rats in litter	At castration	When injections started		Control group	Testicular	Testicular + others
1	2·5	60	—	—	5	26	48	22	175	206	206
2	2·5	60	—	—	5	23	45	22	182	202	194
3	5·0	—	74	—	5	30	52	22	242	219	213
4	?	—	74	—	4	23	65	42	267	270	270
5	5·0	—	74	—	5	30	151	121	430	382	386
6	1·25	—	—	1 mg./10g.	8	27	47	20	207	218	207
7	1·25	—	—	1 mg./10g.	4	23	43	20	—	154	152
8	2·5	—	—	1 mg./ 20 to 100g.	3	24	99	75	242	276	258
9	2·5	—	—	1 mg./ 20 to 100g.	3	26	94	68	289	267	315
10	2·5	—	—	1 mg./ 20 to 100g.	4	26	94	68	312	306	291

Table II. *Average percentage increase for each litter in weight of prostate with seminal vesicles in the group of rats injected with different batches of testicular hormone as compared with that of control uninjected rats.*

No. of batch	Daily dose of c.u. injected	No. of litter	% increase in weight of prostate + sem. vesicles
A. 12	1·25	6	123·0
	1·25	7	No control uninjected rats
76·4	2·5	1	269·5
	2·5	2	204·6
	2·5	8	232·7
	2·5	9	212·0
	2·5	10	211·5
10. 32. 1.	5·0	5	481·0
	5·0	3	173·3
Not marked	?	4	532·0

injected twice a day in half doses, each hormone being injected separately. 24 rats, belonging to 5 litters, were used for the experiments with antuitrin S or prolan and testicular hormone; and 22 rats, belonging to 5 other litters, were used for the experiment with thyroid and testicular hormone. Each litter except No. 7 was divided into 3 groups: Group I, control, untreated rats; Group II, rats injected with testicular hormone only; Group III, rats injected with the same dose of testicular hormone as group II and given in addition antuitrin S

[1] We wish to express our thanks to Messrs Schering Ltd., Messrs Parke Davis and Company and Messrs Bayer Products Ltd. for kindly supplying the preparations.

or antuitrin S and prolan, or thyroid (see Table I). Litter 7, consisting of 4 rats, was divided into two groups only, namely, II and III, without a group of control, untreated rats.

The injections of testicular hormone, antuitrin S and prolan, were all given for 10 consecutive days, but the thyroid-fed rats were given thyroid doses not only during these 10 days of injection but also for 7 days (litters Nos. 6 and 7) or 9 days (litters Nos. 8, 9 and 10) previously, making the total period of thyroid feeding 17 to 19 days. This was done to make the effect of thyroid administration more marked. To save space in Tables II and III only the percentage changes are summarised.

Table III. *Average percentage changes for each litter in weight of organs (calculated per 200 g. of body weight) and in final weight of rats in the group of rats receiving besides testicular hormone other hormones, as compared with that of the group of rats receiving testicular hormone alone.*

No. of litter	Retro-perit-oneal fat	Prostate and seminal vesicles	Penis	Adrenals	Thy-roid	Hypo-physis	Thymus	Liver	Kidney	Spleen	Heart	Final weight of rats
					Testicular hormone and antuitrin S or antuitrin S with prolan.							
1	− 2·9	+ 9·0	− 1·6	− 1·4	− 6·9	+ 6·0	+29·4	− 0·5	+ 0·9	+15·6	− 5·1	± 0
2	−19·6	+48·3	+13·1	+ 8·9	+ 6·7	− 3·8	− 3·2	− 3·9	+12·5	+22·5	+ 8·5	− 4·0
3	−13·5	+40·9	+ 6·4	+ 9·0	+10·7	− 2·1	− 9·2	−18·1	− 0·9	+ 8·0	+ 5·8	− 2·7
4	− 3·3	+10·7	+17·7	+ 3·9	−10·1	− 7·4	−16·3	− 0·7	− 2·0	+20·7	− 4·3	± 0
Average	**− 9·8**	**+27·2**	**+ 8·9**	**+ 5·1**	**+ 0·1**	**− 1·8**	**− 0·2**	**− 5·8**	**− 2·6**	**+16·7**	**+ 1·2**	**− 1·7**
5	+ 1·8	−24·6	+ 3·3	− 8·5	+ 6·8	−19·0	− 9·9	− 7·6	− 2·3	−12·1	−11·9	+ 1·0
					Testicular hormone and thyroid powder.							
6	−17·5	− 5·6	− 0·8	+17·0	−23·5	+13·5	− 9·6	+ 6·8	+27·3	+47·2	+50·6	− 5·0
7	−57·0	− 7·1	+ 3·2	+31·8	−28·8	+10·3	+23·7	+17·1	+35·5	+54·5	+44·9	− 1·3
Average	**−37·3**	**− 6·4**	**+ 1·2**	**+24·4**	**−26·2**	**+11·9**	**+ 7·1**	**+12·0**	**+31·4**	**+50·9**	**+47·8**	**− 3·2**
8	−22·6	− 2·0	−12·0	+ 8·9	−11·7	+15·9	+34·0	+ 3·6	+11·2	+ 3·0	+ 7·6	− 6·5
9	− 6·6	+ 3·8	− 2·8	−23·9	−33·7	−26·6	−24·2	+ 2·5	+ 4·7	+12·4	+11·9	+18·0
10	+ 2·8	+ 5·1	− 3·7	− 2·1	+15·0	+14·5	+28·0	− 1·9	+ 2·8	− 1·4	+ 7·9	− 4·9
Average	**− 8·8**	**+ 2·3**	**− 6·2**	**− 5·7**	**−10·1**	**+ 1·3**	**+12·6**	**+ 1·4**	**+ 6·2**	**+ 4·7**	**+ 9·1**	**+ 2·2**

Simultaneous administration of testicular hormone with antuitrin S or with a mixture of antuitrin S and prolan.

This experiment was performed on litters 1 to 5 (Table I). The percentage increase in weight of the prostate with seminal vesicles in the group of rats injected with testicular hormone alone as compared with that of the control group of uninjected rats is shown in Table II (see litters 1 to 5). Different doses of different batches of this hormone produced an increase in weight of the prostate with seminal vesicles of from 123 to 532 %. As can be seen from Table III, in the rats receiving additional injections of antuitrin S or of antuitrin S and prolan, the effect of the testicular hormone was increased, taking an average of 4 litters of young rats, by 27·2 %, but in one litter of adult rats it was decreased by 24·6 %. The percentage increase reached 48·3 and 40·9 respectively, in litters 2 and 3, in two others it was very small.

Such slight and variable results indicate that in the absence of gonads the gonad-stimulating hormones used in our experiments did not influence the activity of the testicular hormone in a constant and pronounced way. From Table III it is also impossible to find a definite marked effect on the weight of the rats, on fat deposition or on the weight of the other organs investigated.

Simultaneous administration of testicular hormone with thyroid powder.

Litters 6 to 10 were used for this experiment (see Tables I, II and III). Litters 8 to 10 were given 1 mg. thyroid powder per 20 g. of body weight in the preliminary period of thyroid feeding, whilst during the period of simultaneous administration of hormones they were given a much smaller dose of 1 mg. per 100 g. of body weight. Litters 6 and 7 received a comparatively large dose, namely, 1 mg. per 10 g. of body weight during the whole period. We have, therefore, divided these 5 litters into two groups: litters 8–10 and litters 6–7. Only in the latter group receiving large doses were constant definite changes observed; as shown in Table III, these changes were a decrease in fat deposition and in the weight of the thyroids and an increase in the weight of the adrenals, liver, kidneys, spleen and heart. All these changes, however, are typical for rats fed with thyroid powder only; and from the results obtained it is possible only to conclude that testicular hormone injections did not counteract the effects of thyroid administration. The typical effect of testicular hormone on sexual organs was not in any way influenced by thyroid feeding (Table III). Smaller doses of thyroid only produced a much less noticeable thyroid effect and did not alter the effect on the sexual organs.

Summary.

1. The effect of a testicular hormone preparation on the sexual, endocrine and other organs investigated of castrated male rats was not altered to a considerable extent by the additional administration of gonad-stimulating hormones extracted from urine or by feeding with desiccated thyroid powder in the doses given.

2. Testicular hormone preparation did not prevent the appearance of the typical changes produced by thyroid feeding, namely, a decrease in the fat deposition and in weight of thyroids and an increase in the weight of adrenals, liver, kidneys, spleen and heart.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work, and to them our thanks are due.

CCVII. HYDROGENASE.

III. THE BACTERIAL FORMATION OF METHANE BY THE REDUCTION OF ONE-CARBON COMPOUNDS BY MOLECULAR HYDROGEN.

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The production of methane from a 1-carbon compound was first observed by Sohngen in his classical work on methane formation from fatty acids [Sohngen, 1910], calcium formate being quantitatively decomposed to give methane and calcium carbonate. The same culture also effected the reduction of carbon dioxide. It is important to notice that Sohngen worked with mixed cultures which produced methane also from the higher fatty acids. More recently Fischer *et al.* [1931] have obtained from mud a culture (not claimed by them to be pure) which reduces carbon monoxide to methane, the reaction being accelerated by various colloids. These authors suggest that carbon dioxide is an intermediate product, some evidence being adduced for the occurrence of the reaction

$$CO + H_2O \rightarrow CO_2 + H_2.$$

They also showed [1932] that if the ratio of carbon monoxide to hydrogen exceeded one-third, carbon dioxide and methane both resulted, while in higher proportions of hydrogen methane alone was produced.

In the work about to be described we have obtained an organism which we believe to be in pure culture. This differs from the mixed cultures of previous workers in attacking, so far as we have been able to observe, only 1-carbon compounds.

The culture.

The culture used by us was originally obtained from the River Ouse, which had been recently subjected to an influx of fermentable carbohydrate material from a beet-sugar factory and had given a visible fermentation with evolution of gas in the river itself. By subculturing on a medium containing the usual salts [Stephenson, 1930] with 0·5 % formic acid in the form of the sodium salt as the sole source of carbon, and incubating anaerobically, a culture was obtained which decomposed formic acid according to equation (1)

$$4H.COOH \rightarrow CH_4 + 3CO_2 + 2H_2O + 39\,\text{kg. cals.} \qquad \ldots\ldots(1).$$

The culture was continued on this medium for upwards of three years, involving some hundreds of subcultivations, and there seems to be no doubt that growth depends on the decomposition of the formic acid as above and is not due to any extraneous source of carbon. After a large number of subcultures on this medium

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the culture appeared morphologically homogeneous, but very numerous attempts to get growth on agar plates failed, and the earlier experiments here described were carried out with no certainty as to whether we were dealing with a pure culture or with an association of organisms of similar physiological properties. Subsequently we resorted to the single cell technique[1] and by this means obtained a culture descended from one cell. The experiments were then repeated with the pedigree culture, the results obtained being identical with those from the crude culture.

As the physiology of the organism is peculiar and not yet fully worked out, we propose to incorporate its full description in a subsequent paper and to confine ourselves here to the chemical aspect of the subject.

Cultivation of the organism.

In our early experiments we found that very large inoculations (1 cc. in 10) were necessary to get certain growth; later we showed that if the old medium were sterilised by passing through a Seitz filter and added to the new medium at the time of sowing a small inoculation served. Finally we found that the Seitz filtrate could be replaced by sulphide (which it always contained owing to the reduction of sulphate). Our final procedure was as follows. The medium to be inoculated was sterilised by autoclaving; immediately before sowing a solution of sodium sulphide in similar medium readjusted to p_H 7 and sterilised by filtration was added, the concentration of the sulphide being arranged to give a final concentration of 0·035 % $Na_2S, 9H_2O$. Immediately after this addition the culture was inoculated and the anaerobic conditions established; delay results in the rapid oxidation of the sulphide. The culture is able to grow indefinitely without the addition of sulphide, provided the sowings are large enough. This is explained by the fact that the bacteria can reduce the sulphate of the medium to sulphide, as is shown in Exp. 7 on p. 1523. Cysteine and reduced glutathione cannot take the place of sulphide in promoting the growth of the culture.

In order to grow the bacteria in bulk six boiling-tubes each containing 30 cc. of medium were first inoculated; when growth was obtained (8 to 14 days) the whole of the contents of the tubes were sown into one litre of medium. A small quantity of sterile mud results in quicker growth at all stages, but is open to the objection that constituents of the mud may be entering into the subsequent reactions. We have tested this point experimentally, and found that the objection is not valid (see Exp. 8, p. 1524); further evidence is given by the fact that identical results are obtained whether the growth of the bacteria has been aided by mud or not. When the culture was well grown, as shown by vigorous effervescence, it was centrifuged and washed with 0·9 % sodium chloride solution or Ringer's solution with full sterile precautions. The growth from one litre of medium was used for each experiment, but it must be realised that the growth is very scanty as compared with that of most organisms grown on broth.

Apparatus.

This consisted of a bolt-headed flask of about 250 cc. capacity attached to a manometer (see Fig. 1). The method used for sterilisation is fully described in a previous communication [Stephenson and Stickland, 1931, 2].

Exp. 1. The decomposition of formic acid into methane and carbon dioxide. This is the reaction occurring during the growth of the organism on the formate

[1] One of us (M. S.) owes Prof. Morton Kahn of Cornell University and his assistant Mrs Schwarzkopf the warmest thanks for instruction and help in the use of this method.

medium; the quantitative nature of the process was established by the use of washed suspensions as follows.

Into the flask were put 10 cc. of phosphate buffer, p_H 6·4, 10 cc. of 0·25 *N* sodium formate, 10 cc. bacterial suspension (from one litre of medium) and 10 cc. of water. The flask was then attached to the manometer as previously described; nitrogen passed over heated copper was then introduced and the process repeated. After filling with nitrogen for the second time the manometer was connected to the flask by turning the tap T_1 and the apparatus placed at 37°. When the manometer reading was constant (after 1½–2 hours) the initial reading was taken, together with the temperature and pressure. When the reaction was complete (4–5 days), *i.e.* when no further change of pressure took place, the final readings of manometer, temperature and barometer were taken, and a sample of gas was removed through tap T_3 for analysis.

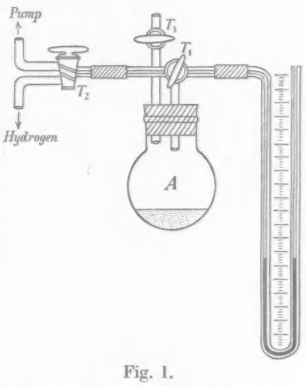


Fig. 1.

The carbon dioxide remaining bound in the solution as bicarbonate was estimated by acidifying an aliquot part and blowing the carbon dioxide into standard baryta by means of a current of air. The complete figures for one experiment are given in Table I and the results of three similar experiments in Table II.

Table I.

Formic acid decomposed (mg.)	115
Volume of gas space of flask (cc.)	168
Final temperature	35°
Final barometric pressure (mm.)	763·0
Final manometer reading (mm.)	+108·0
Final composition of gas	H_2 0·0 %; CO_2 6·35 %; CH_4 8·7 %; N_2 (by difference) 84·95 %
Final CO_2 in solution	28·2 cc. of 0·0977 *N* acid
Volumes of gas formed at N.T.P. (cc.)	CO_2 41·1; CH_4 14·1

Table II.

	Formic acid decomposed (millimols)	CO_2 formed (millimols)	CH_4 formed (millimols)	Formic acid / 4 (millimols)	CO_2 formed / 3 (millimols)
1	2·00	1·53	0·46	0·50	0·51
2	2·00	1·47	0·45	0·50	0·49
3*	2·50	1·83	0·63	0·625	0·61

* Indicates experiment with pure culture.

Thus a washed suspension of the culture decomposes formic acid quantitatively in accordance with equation (1) (p. 1517). (The last two columns in Table II are added to show the extent of the agreement more clearly.)

Exp. 2. The reduction of carbon dioxide by hydrogen to methane. We next investigated whether the culture responsible for the reaction described in the last section could also effect the reduction of carbon dioxide to methane by molecular hydrogen, as Sohngen's did. The same apparatus was used and in the flask were placed 10 cc. buffer p_H 7·0, 10 cc. bacterial suspension and 20 cc. water. After connection to the manometer, the flask was evacuated; pure carbon dioxide from a Kipp's apparatus was then let in to a pressure of about 60 mm.

(bringing the p_H of the solution to 6·5, the optimum), and this pressure was read off accurately on the manometer attached to the pump; the flask was then completely filled with pure hydrogen, and the tap turned to connect it with its own manometer. Finally, the manometer readings, the barometric pressure and the room temperature were taken; from these data the absolute volumes of carbon dioxide and hydrogen in the flask could be calculated. The whole apparatus was then placed at 37°, and when the uptake of gas had finished (in about 6 days) the temperature and manometer and barometer readings were again taken and a sample of gas withdrawn for analysis. The complete results of a typical experiment are given in Table III, and the condensed results of four experiments in Table IV.

Table III.

Original pressure of CO_2 (mm.) 65·5
Volume of gas space in flask (cc.) 176
Final composition of gas CO_2 0·0 %; H_2 86·7 %; CH_4 13·5 %

	Initial	Final	Difference
Temperature	20°	36°	
Barometric pressure (mm.)	757·5	761·5	
Manometer reading (mm.)	+19·5	−198·5	
Volume of CO_2 at N.T.P. (cc.)	14·2	0·0	−14·2
Volume of H_2 at N.T.P. (cc.)	150·0	92·0	−58·0
Volume of CH_4 at N.T.P. (cc.)	0·0	14·3	+14·3

Table IV.

	Hydrogen used (millimols)	Carbon dioxide used (millimols)	Methane formed (millimols)	$\frac{\text{Hydrogen used}}{4}$ (millimols)
1	2·59	0·64	0·64	0·65
2	2·56	0·63	0·61	0·65
3	2·85	0·70	0·69	0·71
4*	4·48	1·00	0·97	1·12

* Indicates experiment with pure culture.

The theoretical equation is

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{.....(2),}$$

and the results obtained agree with this, so we must conclude that the same culture which decomposes formic acid to methane and carbon dioxide is also capable of reducing carbon dioxide to methane by means of molecular hydrogen.

It will be remembered that Sohngen carried out the fermentation of a number of fatty acids in the presence of hydrogen and obtained greater yields of methane with uptake of hydrogen. We therefore allowed our culture to decompose formate in an atmosphere of hydrogen, and estimated the methane formed and the hydrogen used.

Exp. 3. The reduction of formic acid with hydrogen. The same method was used as in Exp. 1, but only 5 cc. of 0·25 *N* formate were taken, and the apparatus was filled with hydrogen. After about six days there was no further movement of the manometer, and the usual readings were taken, a sample of gas was withdrawn and the residual formic acid estimated. The results of three experiments are given in Table VI, with complete details of one of them in Table V.

The equation is

$$H.COOH + 3H_2 \rightarrow CH_4 + 2H_2O \qquad \text{.....(3).}$$

From these experiments it is clear that if the change of formic acid into methane is carried out in an atmosphere of hydrogen no carbon dioxide is produced, but the carbon of the formic acid is completely reduced to methane.

Table V.

Formic acid decomposed (mg.)	56·2		
Volume of gas space in flask (cc.)	170		
Final composition of gas	H_2 72·6 %; CH_4 27·4 %		
	Initial	Final	Difference
Temperature	36°	36°	
Barometric pressure (mm.)	767·0	743·5	
Manometer reading (mm.)	+ 36·0	− 227·5	
Volume of H_2 at N.T.P. (cc.)	150·0	69·0	− 81·0
Volume of CH_4 at N.T.P. (cc.)	0·0	26·0	+ 26·0

Table VI.

	Formic acid decomposed (millimols)	Hydrogen used (millimols)	Methane formed (millimols)	$\frac{\text{Hydrogen used}}{3}$ (millimols)
1	1·22	3·62	1·16	1·21
2	1·25	3·91	1·22	1·30
3*	1·11	3·36	1·16	1·12

* Indicates experiment with pure culture.

Exp. 4. The reduction of carbon monoxide by hydrogen to methane. The usual phosphate buffer and cell suspension were placed in the flask in a total volume of 40 cc., and the flask was evacuated and filled with a gas mixture containing about 95 % hydrogen and 5 % carbon monoxide, accurately analysed in the Haldane apparatus. The gas was freed from oxygen first by heating a coil of platinum wire in it and then by bubbling it through alkaline pyrogallol on its way to the flask; the former of these two treatments reduced the oxygen content below that detectable in the Haldane apparatus, *viz.* 0·02 %. When the apparatus was incubated at 37°, the gas volume diminished rather slowly, and after several weeks the hydrogen uptake stopped considerably short of the theoretical amount. At the end of the experiment the usual readings were taken, and a sample of gas was withdrawn for estimation of hydrogen, carbon monoxide and methane.

The distinction between carbon monoxide and methane in this mixture depended on the estimation of the oxygen remaining after burning the gas, and as the total amount of $(CO + CH_4)$ was only roughly 6 % of the gas, and the analyses had to be done on samples of 0·8 cc., the results were less accurate than usual. These particular gas samples were always analysed in triplicate, and the values agreed as well as could be expected. Further, a mixture of carbon monoxide and hydrogen, containing no methane, on analysis by this method, gave values for methane between + 0·1 % and − 0·5 %.

Table VII. *Analyses of mixture of H_2 and CO, to test accuracy of analyses of mixtures of H_2, CO and CH_4.*

		(1) %	(2) %			(1) %	(2) %
(*a*) By ordinary method	H_2	80·9	80·3	(*b*) By special method	H_2	81·3	80·8
	CO	19·1	19·4		CO	19·2	20·0
		100·0	99·7		CH_4	0·1	− 0·5
						100·6	100·3

The results of experiments on the reduction of carbon monoxide are given in Tables VIII and IX. The equation representing the reaction is

$$CO + 3H_2 \rightarrow CH_4 + H_2O \qquad \text{.....(4),}$$

and the results are sufficiently close to show that this is actually the reaction that takes place; the experimental error is greater than in the previous experiments.

Table VIII.

Volume of gas space in flask (cc.)	170		
Initial composition of gas	H_2 91·6 %; CO 7·3 %; CH_4 0·0 %.		
Final composition of gas	H_2 90·8 %; CO 3·6 %; CH_4 4·1 %.		
	Initial	Final	Difference
Temperature	36°	37°	
Barometric pressure (mm.)	755·0	751·0	
Manometer reading (mm.)	+ 82·0	− 15·0	
Volume of H_2 at N.T.P. (cc.)	143·5	123·2	−20·3
Volume of CO at N.T.P. (cc.)	11·4	4·9	− 6·5
Volume of CH_4 at N.T.P. (cc.)	0·0	5·6	+ 5·6

Table IX.

	Hydrogen used (millimols)	CO reduced (millimols)	CH_4 produced (millimols)	$\frac{H_2 \text{ used}}{3}$ (millimols)
1	0·91	0·29	0·25	0·30
2*	0·61	0·18	0·175	0·20

* Indicates experiment with pure culture.

Exp. 5. The reduction of formaldehyde by hydrogen. Formaldehyde is far too toxic for use in our apparatus, as the smallest amount the reduction of which could be measured would be sufficient to kill the bacteria and destroy their enzymes. The only possibility was to use some very slightly dissociated compound of formaldehyde, such as hexamethylenetetramine, and this was done.

10 cc. of 0·63 % hexamethylenetetramine were placed in the flask with the usual buffer and bacterial suspension in a total volume of 40 cc., and the apparatus was filled with hydrogen and incubated at 37°. Hydrogen uptake continued for two to three weeks, when the gas was analysed and the remaining hexamethylenetetramine estimated. This was done by converting the hexamethylenetetramine into formaldehyde by treatment with dilute sulphuric acid, allowing the aldehyde to react with sodium bisulphite (in excess) and titrating the bound sulphite with standard iodine in the usual way. The method is unsatisfactory, owing to the fact that the reaction of hexamethylenetetramine with dilute acids produces, besides formaldehyde and ammonia, methylamine, dimethylamine and other products; too low results in the initial and final hexamethylenetetramine estimations would partly account for the low value for "formaldehyde reduced."

The results (Tables X and XI) are sufficient to show that the reaction

$$H.CHO + 2H_2 \rightarrow CH_4 + H_2O \qquad \ldots\ldots(5)$$

does really occur.

Table X.

Formaldehyde disappeared (mg.)	40		
Volume of gas space in flask (cc.)	162		
Final composition of gas	H_2 70·1 %; CH_4 29·8 %		
	Initial	Final	Difference
Temperature	35°	37°	
Barometric pressure (mm.)	764·0	769·0	
Manometer reading (mm.)	+ 81·0	−146·0	
Volume of H_2 at N.T.P. (cc.)	151·4	73·5	−75·9
Volume of CH_4 at N.T.P. (cc.)	0·0	32·1	+32·1

Table XI.

	Formaldehyde reduced (millimols)	Hydrogen used (millimols)	Methane formed (millimols)	$\frac{H_2 \text{ used}}{2}$ (millimols)
1	1·34	3·39	1·43	1·69
2*	0·215	0·46	0·20	0·23

* Indicates experiment with pure culture.

Exp. 6. The reduction of methyl alcohol to methane by hydrogen. This was carried out in the usual way except that special precautions were taken to avoid loss of methyl alcohol vapour during the evacuation of the flask. 10 cc. of buffer at p_H 6·3, 7 cc. of water and 20 cc. of bacterial suspension were placed in the flask, which was then twice evacuated and filled with hydrogen, leaving at the end a diminished pressure of about 20 mm. of mercury. 1 cc. of the methyl alcohol solution was then added through the tap, care being taken that no air was admitted, and washed down twice with 1 cc. of water, and the flask was then completely filled with hydrogen and the whole apparatus incubated at 37°. Theoretically, if the reaction taking place is

$$CH_3OH + H_2 = CH_4 + H_2O \qquad \ldots\ldots(6),$$

no change of pressure should occur. The results are given in Tables XII and XIII; in Exps. 1 and 2 excess of methyl alcohol was present, and the amount left was not estimated, but the agreement between hydrogen used and methane formed is good, and in Exp. 3 the methyl alcohol was completely accounted for as methane.

Table XII.

Volume of gas space in flask (cc.)	180		
Final composition of gas	H_2 91·2 %; CH_4 8·6 %		
	Initial	Final	Difference
Temperature	34°	36°	
Barometric pressure (mm.)	753·0	761·0	
Manometer reading (mm.)	+ 74·0	+ 73·0	
Volume of H_2 at N.T.P. (cc.)	165·5	151·0	−14·5
Volume of CH_4 at N.T.P. (cc.)	0·0	14·2	+14·2

Table XIII.

	Methyl alcohol reduced (millimols)	Hydrogen used (millimols)	Methane formed (millimols)
1	—	0·65	0·64
2	—	0·30	0·29
3*	0·62	0·54	0·59

* Indicates experiment with pure culture.

Exp. 7. The reduction of sulphate to sulphide by hydrogen. This experiment was carried out in the same way as those previously described [Stephenson and Stickland, 1931, 2]. The results show that the methane-forming culture can also carry out the reaction

$$H_2SO_4 + 4H_2 = H_2S + 4H_2O.$$

The experimental results are summarised in Table XIV.

Table XIV.

Na_2SO_4 reduced (millimols)	Hydrogen used (millimols)	$\frac{H_2 \text{ used}}{4}$ (millimols)	Hydrogen sulphide formed (millimols)
0·42	1·60	0·40	0·35

Exp. 8. Control. The bacteria from a litre of medium together with the mud of the medium on which they had grown were placed in the manometer apparatus as usual, but without the addition of any carbon compound. After incubation with hydrogen for fourteen days no methane was found in the gas, showing that the results of the quantitative experiments are not invalidated by the presence of mud. This result received confirmation in experiments in which various 2-carbon compounds were added and in which no methane was formed.

Experiments on reaction velocity.

Of the six reactions described in which carbon compounds are converted into methane, the three involving carbon dioxide and formic acid proceed easily and rapidly and are completed in our manometer apparatus in a few days. The reductions of carbon monoxide, formaldehyde and methyl alcohol, on the other hand, are relatively very slow and are usually incomplete in twenty-one days, even with low initial concentrations. This difference of velocity has been confirmed by experiments in Barcroft apparatus, in which a thick suspension of bacteria (pure culture) was allowed to act on carbon dioxide, formic acid, hexamethylenetetramine and methyl alcohol in an atmosphere of hydrogen. The substrate concentration was in each case $N/30$, and the p_H 6·5; for carbon dioxide the p_H was adjusted by using a mixture of 50 % hydrogen and 50 % carbon dioxide in equilibrium with $N/30$ sodium bicarbonate. The results showed that formic acid and carbon dioxide are reduced at roughly the same rate, while hexamethylenetetramine is reduced at a rate too slow for measurement in the conditions of such experiments. Values for the rate of reaction in terms of the dry weight of bacteria present cannot be given, as the suspension of bacteria is always mixed with a small amount of mud which makes estimations of dry weight or nitrogen content useless.

In these experiments the reaction curve starts immediately in a straight line, which—especially in the case of a very slow-growing organism—precludes the idea that cell multiplication is interfering appreciably with the results.

Experiments on the course of the reactions.

The mechanism involved in the production of methane from these compounds must now be considered. The decomposition of formic acid into hydrogen and carbon dioxide ($H.COOH \rightarrow H_2 + CO_2$) is a common property of many organisms and has recently been shown to be due to a particular enzyme (formic hydrogenlyase) distinct from formic dehydrogenase catalysing the reaction $H.COOH \rightarrow 2H + CO_2$ [Stephenson and Stickland, 1932]. An organism possessing this enzyme together with hydrogenase (activating molecular hydrogen) [Stephenson and Stickland, 1931, 1] and at the same time capable of reducing carbon dioxide might well bring about reaction (1) (p. 1517) in a stepwise manner, by the preliminary decomposition of formic acid into carbon dioxide and hydrogen, followed by a reduction of carbon dioxide by hydrogen as in reaction (2) (p. 1520). Our organism possesses hydrogenase, as is shown by the reduction of methylene blue by hydrogen with a suspension of washed cells:

	Reduction time (min.)
1 cc. 1/5000 methylene blue, p_H 6·3, *in vacuo*	>60
1 cc. 1/5000 methylene blue, p_H 6·3, in hydrogen	6

Moreover, we have obtained evidence for the presence of formic hydrogenlyase; a series of test-tubes containing Durham tubes and formic inorganic medium was

sown in the usual way and gas withdrawn from the Durham tubes for analysis at different stages in the growth of the culture. It is seen from the figures in Table XV that in the early stages hydrogen as well as methane was present, but that if the reaction was allowed to continue methane alone was the final product.

Table XV.

Amount of gas (mm.)	Composition of gas	
	CH_4 %	H_2 %
2	64	36
6	86	14
7	92	8
8	71	29
21	98·5	1·5
31	99·5	0·5

The extent of growth of the culture was measured by the length of the column of gas in the Durham tube, given in the Table in mm.

The same conclusion was reached from experiments carried out in Barcroft manometers. A thick cell suspension was shaken in this apparatus with $M/10$ formate in buffer at p_H 6·5 in an atmosphere of nitrogen; the results showed a brief rapid evolution of gas followed by a slower prolonged evolution. When the gas in the cups was analysed at the end of the first period it was found to contain (besides nitrogen) mainly hydrogen and carbon dioxide with a little methane; after further reaction had taken place the analysis showed decreased hydrogen and carbon dioxide and increased methane.

Analysis of gas in Barcroft cups.

	Total evolution mm.³	H_2 %	CO_2 %	CH_4 %
After 1 hour	448	0·9	1·1	0·1
After 26 hours	1666	0·6	0·3	3·9

This suggests that formic acid undergoes a preliminary decomposition to carbon dioxide and hydrogen, and methane is then synthesised by reaction (2) (p. 1520). This would involve, not a direct reduction of formic acid, but a reduction by excess hydrogen of the carbon dioxide produced in the first reaction. It must be admitted, however, that we are offering here a plausible hypothesis and no proof.

In respect of reactions (4), (5) and (6) (pp. 1521, 1522 and 1523 respectively) we are in the dark. Possibly a preliminary reduction of all three initial compounds to formic acid and a decomposition of this first to carbon dioxide and hydrogen and thence to methane is as plausible as any. It should be noted that recent workers [Fischer *et al.*, 1932], have suggested that in the case of carbon monoxide a decomposition of water takes place thus

$$CO + H_2O \rightarrow CO_2 + H_2.$$

Their evidence rests on the appearance of 13·4 cc. of hydrogen in a bacterial decomposition of 1100 cc. of carbon monoxide in the presence of mud, giving 323 cc. of methane and 724 cc. of carbon dioxide. Without a control experiment

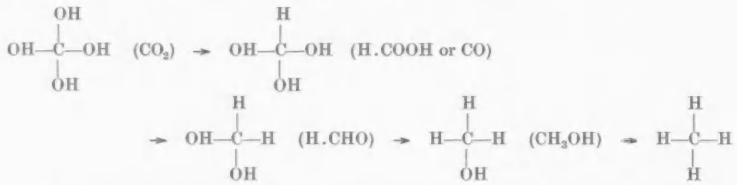
in nitrogen no evidence exists to decide whether carbon monoxide is transformed to methane *via* this reaction

$$CO + H_2O \rightarrow CO_2 + H_2$$

or by hydration to formic acid

$$CO + H_2O \rightarrow H.COOH.$$

As an alternative it is possible that formic acid is a stage in the reduction of carbon dioxide to methane by a stepwise process thus:



On this view formic hydrogenlyase would be competing with the enzyme concerned in the reduction of formic acid, and the hydrogen produced by its action in the initial stages would be finally used up again in the reduction.

The reduction of other carbon compounds.

We have shown that five simple 1-carbon compounds are reduced by our organism to methane. On the other hand the following compounds were not attacked: acetic, propionic, butyric and caprylic acids (which were all decomposed to methane and carbon dioxide by Sohngen's mixed culture), ethyl alcohol, acetaldehyde and glucose. These compounds were tested in conditions where they formed the sole source of carbon for the organism, and also in broth cultures; in the former case no growth occurred, and in the latter the bacteria grew on the broth but failed to produce any methane from the added compound. In addition acetic acid, acetaldehyde and ethyl alcohol were tested in the manometer apparatus, with washed suspensions, with negative results. As far as we have tested, therefore, only compounds with one carbon atom are reduced by our culture.

Summary.

1. An organism has been isolated by the single cell technique which is able to live anaerobically on an inorganic medium with formate as sole source of carbon.

2. The formic acid is decomposed as follows:

$$4H.COOH = CH_4 + 3CO_2 + 2H_2O + 39 \text{ kg. cals.}$$

This reaction has been shown to occur in two stages, *viz.*

$$(1)\ H.COOH = H_2 + CO_2,$$

and

$$(2)\ 4H_2 + CO_2 = CH_4 + 2H_2O,$$

and is therefore the work of two enzymes, formic hydrogenlyase (1) and hydrogenase (2) with an additional mechanism for the activation of carbon dioxide as a hydrogen acceptor.

3. The organism (tested in washed suspensions) reduces the following 1-carbon compounds to methane by means of molecular hydrogen (*i.e.* hydro-

genase reactions): carbon dioxide, formic acid, carbon monoxide, formaldehyde (as hexamethylenetetramine) and methyl alcohol.

4. No compounds so far tested containing more than one carbon atom are reduced by this organism with production of methane.

We take this opportunity of thanking Sir Frederick Hopkins for his interest in this work.

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CCVIII. HYDROGENLYASES.

III. FURTHER EXPERIMENTS ON THE FORMATION OF FORMIC HYDROGENLYASE BY *BACT. COLI.*

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(*Received August 28th, 1933.*)

In a recent paper [Stephenson and Stickland, 1932] we showed that a culture of *Bact. coli* grown in Roux bottles on tryptic broth was devoid of the enzyme formic hydrogenlyase, while a culture grown in similar circumstances with addition of formate to the medium possessed this enzyme. Yudkin [1932] pointed out that there are two possible explanations of this phenomenon. (1) The difference might be due to a process of "natural selection," or (2) it might be due to a direct chemical action of the formate and broth, stimulating the formation of a new enzyme in the cells. He decided against the former theory, his evidence being (1) the probable uselessness to *Bact. coli* of being able to decompose formic acid anaerobically, and (2) the fact that when a culture (in this case of *Bact. freundii*) grown for many generations on broth containing formic acid was resown on to plain broth, it completely lost its formic hydrogenlyase during sixteen hours' growth (about ten generations). We propose now to offer some further evidence on this point.

For "natural selection" to operate, the formate must have either a beneficial or a harmful effect on the bacteria; in the former case those cells able to profit by the decomposition of formic acid, and in the latter those able to rid themselves of it by decomposition, would have an advantage. In either case, the addition of formate to a young, rapidly growing culture of *Bact. coli* would be expected to have some effect on the subsequent growth rate, and this point was tested experimentally.

Two one-litre flasks containing 500 cc. of tryptic broth were sterilised and brought to 37° by incubation overnight. Each flask was then sown with one drop of a young broth culture of *Bact. coli*, and a sample of each taken for counting. At intervals of 1, 2, $2\frac{1}{2}$, 3, $3\frac{1}{2}$ and 4 hours further samples were withdrawn for counting, to establish the growth rate, and at 4 hours 10 cc. of sterile 25 % formic acid as sodium salt were added to one flask (giving a concentration of 0·5 %) and 10 cc. of sterile water to the other, both solutions having been previously warmed to 37°. After the additions samples were again taken at intervals of 5 minutes, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$ and $3\frac{1}{2}$ hours; the counting was carried out by Wilson's viable count method [1922]. The results, shown graphically in Fig. 1, show that the addition of sodium formate had no effect on the growth rate of the culture, so we must conclude that the mode of action of the formate is not "natural selection."

[1] Beit Memorial Research Fellow.

The second alternative, *viz.*, a direct chemical action of the formate and broth on the cells, was therefore provisionally accepted, and we proceeded to try to find further experimental support of this view.

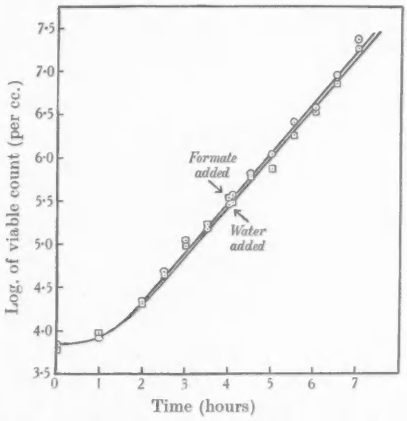

Fig. 1. ⊡ Exp. (formate added); ⊙ Control (water added).

Growth and enzyme formation.

Our next step was to determine how many generations and how much time were required for the appearance of the enzyme after addition of the formate. This could not be done while growth was still in the logarithmic phase, as at that stage the concentration of bacteria was insufficient for enzyme determinations; the youngest culture from which sufficient bacteria for testing could be obtained by centrifuging was ten hours old, and a culture of this age was consequently used.

A four-litre flask containing three litres of medium, fitted with a rubber stopper carrying a wide glass inlet tube and a siphon, was sterilised and sown with two drops of an eight-hour old culture of *Bact. coli*. Ten hours later, when growth was just visible, a sample of 500 cc. was siphoned off into sterile centrifuge-tubes, and 50 cc. of sterile 12·5 % formic acid (as sodium salt) were added to the remaining culture through the wide tube in the stopper, giving a final concentration of 0·5 % formic acid. Further samples of 500 cc. were run off into sterile centrifuge-tubes at intervals of 1, 2, 3 and 4 hours after the addition of the formate. Each sample as it was obtained was treated as follows. The cups were cooled to roughly 10° by immersion in ice, and a small sample (about 1 cc.) was taken into a sterile dry tube and diluted immediately for viable counting. The remainder was centrifuged and washed once with sterile Ringer's solution. The washed suspensions thus obtained were tested for formic hydrogenlyase (as described by Stephenson and Stickland [1932], and with the help of nitrogen estimations on the bacteria values for Q_{H_2} were calculated. The results of one experiment are given in Table I.

It will be seen that the formic hydrogenlyase had appeared in less than one hour after the addition of the formate, while the viable count had increased by only 18 %, and the Q_{H_2} had reached its maximum after two hours with an increase of only 34 % in the viable count. It appears, therefore, that the enzyme formation

Table I.

Time after addition of formate (hours)	Viable count (10^7 per cc.)	Q_{H_2}
0	5·0	0
1	5·9	450
2	6·7	710
3	8·0	650
4	7·7	650

reaches its maximum during considerably less than one generation time, which fact makes it impossible for the phenomenon to be one of "natural selection."

It might be objected that viable counts in a relatively old culture do not give a true indication of the amount of cell division taking place, as a rapid cell division might be balanced by a high death rate. This objection could be met by using the method of total counting, which would detect any increase of cell numbers with an error of 5 %. This method of counting was used in all subsequent experiments, and at the same time a simpler method was devised for following the course of the production of the enzyme.

The course of the production of formic hydrogenlyase.

A pure suspension of *Bact. coli* containing no formic hydrogenlyase was prepared by growing a culture on plain broth in Roux bottles, centrifuging and washing aseptically. Barcroft cups were sterilised by standing them in chromic acid overnight, washing them six times with sterile distilled water and plugging them with sterile cotton wool plugs from test-tubes; they were dried by leaving them in an incubator for a few hours. The apparatus was set up with 3 cc. of water in the left-hand cup and the following sterile solutions in the right-hand cup:

Tryptic broth (three times normal strength) p_H 7·0 ...	1·0 cc.
Sodium formate 1·0 *M*	0·3 cc.
Phosphate buffer 0·2 *N*, p_H 7·0	0·5 cc.
Water	0·2 cc.

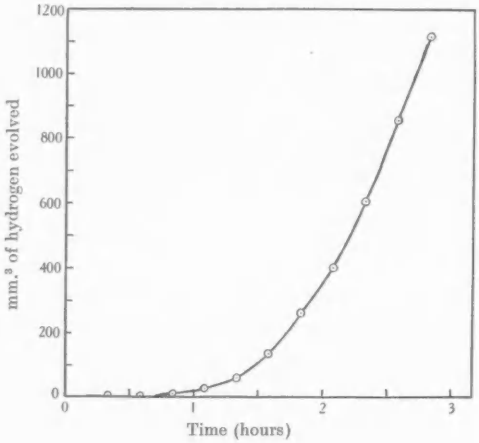

Fig. 2.

1 cc. of a suitably diluted bacterial suspension was then added and the apparatus filled with nitrogen as usual and placed in the bath at 40°.

In Fig. 2 the result of a typical experiment is given, the volume of hydrogen evolved being plotted against time in the usual way, while in Fig. 3 the same experiment is plotted with velocities of hydrogen evolution as ordinates.

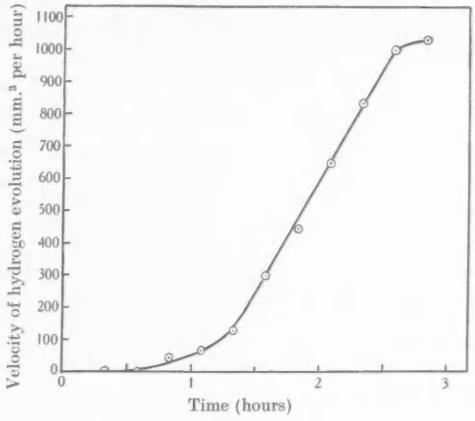

Fig. 3.

Table II.

No.	Duration of exp. (hours)	Total count 10^8/cc. Initial	Total count 10^8/cc. Final	Increase (%)	Q_{H_2} Initial	Q_{H_2} Final
1	$2\frac{1}{2}$	1·14	1·19	0	0	3950
2	2	5·4	4·9	0	0	3900
3	$3\frac{1}{2}$	1·17	1·12	0	0	3500
4	$1\frac{1}{2}$	2·6	2·6	0	0	2160
5	$3\frac{1}{2}$	1·6	1·5	0	0	1900
6	$5\frac{1}{2}$	1·5	1·5	0	0	1670
7	$4\frac{1}{2}$	2·1	2·1	0	0	1600
8	3	4·3	4·45	0	0	1400
9	3	2·5	2·5	0	0	1400
10	3	5·4	5·2	0	0	1200
11	$2\frac{1}{2}$	2·6	2·5	0	0	1120
12	$3\frac{1}{2}$	7·6	7·9	0	0	870
13	3	7·6	7·6	0	0	770
14	3	9·3	9·2	0	0	760
15	2	1·7	1·7	0	0	750
16	$2\frac{1}{2}$	3·2	3·2	0	0	720
17	3	1·14	1·24	9	0	4850
18	3	3·2	3·6	12	0	900
19	3	1·14	1·39	20	0	3600
20	$4\frac{1}{2}$	2·3	3·15	40	0	1200
21	3	3·2	4·8	50	0	560
22	$3\frac{1}{2}$	1·7	3·0	95	0	1240
23	4	1·7	4·0	120	0	1210

It will be observed that, as would be expected, the initial velocity is zero, and after a latent period the velocity begins to increase. The rate of increase of velocity becomes linear and remains so until the maximum is reached; this may

be interpreted as showing that the synthesis of formic hydrogenlyase is a linear reaction.

In a large number of such experiments a total count was carried out, both on the initial suspension of *Bact. coli* and on the contents of the Barcroft cup after the maximum velocity had been reached; the results are given in Table II. It will be seen that in the majority of the experiments no growth (*i.e.*, less than 5 % increase in total count) took place, while the value of Q_{H_2} increased from 0 to 720–3950, and in the remaining cases (7 out of 23) slight growth (never more than 120 % increase) was found with a similar increase in Q_{H_2}.

Discussion.

The experiments on the growth rate of *Bact. coli* after addition of formate to a young culture, and on the appearance of formic hydrogenlyase after such an addition, are sufficient to show that "natural selection" plays no part in the formation of the enzyme. From the experiments in the Barcroft apparatus just described further conclusions can be drawn. In presence of formate alone no formic hydrogenlyase is developed, but in presence of formate and tryptic broth, after a latent period, the appearance of formic hydrogenlyase proceeds proportionally to the time, while no cell division is taking place. It seems reasonable to deduce from this that enzyme formation is simply a chemical reaction between the medium and the cells. Further work is being carried out to try to elucidate the nature of the reaction.

Summary.

1. This work aims at deciding whether the enzyme formic hydrogenlyase appears in *Bact. coli* by a process of "natural selection" or in some other way.

2. Addition of sodium formate to a young culture of *Bact. coli* has no effect on the subsequent growth rate.

3. The enzyme can be produced by the bacteria while no cell division is taking place, and this production proceeds linearly.

4. The deductions made are that (1) the operation of "natural selection" is impossible, and (2) the formation of formic hydrogenlyase is probably a chemical reaction between the medium and the cells.

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CCIX. NOTE ON THE INCIDENCE OF DERMATITIS AMONG RATS DEPRIVED OF VITAMIN B_2.

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(*Received August 31st, 1933.*)

THE most consistent sign of a deficiency of vitamin B_2 in rats is failure of growth, but in addition to this an inflammatory dermatitis, considered by some to be analogous to human pellagra, may develop. This dermatitis, however, is irregular in occurrence; some authors have not observed it in any of their animals, others in only a proportion. Various reasons have been put forward to account for this irregularity. It has been suggested that in order to obtain symptoms constantly absolute deprivation of vitamin B_2 must be ensured [Chick and Roscoe, 1928], or, alternatively, that small amounts of vitamin B_2 must be supplied [Sherman and Sandels, 1931]; also that a seasonal variation in incidence of symptoms occurs [Leader, 1930]. Finally the theory has been advanced that the dermatitis is due to lack of some dietary factor, other than that promoting growth, the distribution of which is not as yet understood [Chick and Roscoe, 1928; Sure and Smith, 1930–31]. Kuhn *et al.* [1933] have in fact, stated that vitamin B_2 can be separated into two factors one of which is a skin factor (vitamin H).

In this paper data are given concerning the incidence of dermatitis among the vitamin B_2-deficient animals observed in this laboratory during the past seven years. In addition, results of experiments are reported, in which attempts were made by variations of the diet to influence the development of dermatitis.

EXPERIMENTAL.

Young rats, shortly after weaning, and weighing 30–40 g., were started on the deficient diet. This consisted of caseinogen 20 parts, rice starch 60, salt mixture[2] 5 and hardened cottonseed oil 15, mixed with an equal weight of water and steamed for 3–5 hours. Cod-liver oil was given separately each day. Vitamins B_1 and B_4 were given as a daily dose of a concentrate from yeast, prepared according to the method described by Kinnersley *et al.* [1933]. The presence of vitamin B_4 in this concentrate was assumed, as extraction of the charcoal was carried out with acid alcohol, in which process both vitamins B_1 and B_4 are stated to be eluted [Reader, 1929].

The type of dermatitis observed in this laboratory in rats deprived of vitamin B_2 has been described by Chick and Roscoe [1927] and is essentially the same as that originally described by Goldberger and Lillie [1926], with the exception that, whereas the lesions observed by them were stated to be bilaterally symmetrical, that has not always been the case with those observed here. Some authors report that the only lesions they obtain as a result of deprivation of

[1] Beit Memorial Research Fellow.

[2] Salt Mixture No. 185. (McCollum *et al.*, 1917.)

vitamin B_2 are a scurfy condition with loss of hair. The latter condition is frequently observed among our vitamin B_2-deficient animals but for the purpose of the present study has not been counted as dermatitis.

In working out the proportion of rats developing dermatitis, only those that survived for more than 10 weeks without symptoms have been counted as negatives.

(1) *Number of rats developing dermatitis.* During the past seven years 108 out of the 191 rats on the vitamin B_2-deficient diets have developed dermatitis, an incidence of 57 %. The figures for each of the seven years were: 14/21, 16/26, 23/30, 6/20, 9/21, 16/24 and 24/49.

(2) *Time taken for dermatitis to develop.* The average time during which the rats received the deficient diet before symptoms developed was 10 weeks; in 32 % of the 108 cases the dermatitis occurred after 9–11 weeks of depletion.

(3) *Seasonal variation in the occurrence of symptoms.* The development of dermatitis in the above 108 rats has shown no significant relation to the time of year. Thus 55 % of the rats born in the quarter January-March developed dermatitis, 45 % of those born in April–June, 61 % of those born in July–September and 62 % of those born in October–December. Nor was the period of development of symptoms longer at any one time of year; during the same quarters the average time was 10, 9, 11 and 10 weeks respectively. The experiments were evenly distributed over these periods.

(4) *Weight increase of vitamin B_2-deficient rats.* Considerable variations in weight increase occurred among the animals receiving the same vitamin B_2-deficient diet. The degree of growth, however, did not appear to bear any relation to the occurrence of dermatitis. Thus the average growth during the first five weeks on the deficient diet was 17 g. for the animals which developed dermatitis, 16 g. for those which did not. Nor was a greater weight increase associated with delay in occurrence of symptoms. Rats developing dermatitis after 0–5, 5–10 or 10–15 weeks of depletion had increased in weight by an average of 23, 18 and 15 g. respectively in the first 5 weeks of the observation. This would seem to indicate that those which grew best were most liable to succumb, but the variations in growth were so great (− 3 g. to + 37 g. in 5 weeks) that the above differences in averages were not significant. After the animals had developed dermatitis, growth ceased, as was to be expected since they were then definitely sick.

(5) *The influence of diet.* At first it was thought necessary to use a highly purified caseinogen in the diet in order to induce dermatitis [Chick and Roscoe, 1928]. Later, as the result of papers by Coward, Key and Morgan [1929] and Coward, Key, Morgan and Cambden [1929] showing that unpurified caseinogen did not contain appreciable amounts of either vitamin B_1 or B_2, diets were tested which contained the same (B.D.H. "light white casein") before purification. Substitution of this less pure material for the purified product did not cause any increase in the growth of rats receiving small amounts of either vitamin B_1 or B_2, nor was it found to influence the incidence of neuritis in vitamin B_1-deficient rats or of dermatitis in vitamin B_2-deficient rats.

The results obtained using three different kinds of caseinogen were as follows.

(i) *Glaxo purified caseinogen (physiological caseinogen, AB).* 13 out of 22 rats (64 %), developed dermatitis after an average depletion period of 8 weeks.

(ii) *Lister Institute purified caseinogen.* 61 out of 107 rats (57 %), developed dermatitis after an average depletion period of 11 weeks.

(iii) *"Light white casein" unpurified.* 36 out of 54 rats (67 %), developed dermatitis after an average depletion period of 9 weeks.

There was thus no significant difference between the effects of diets containing these different caseinogens upon the incidence of dermatitis. The increase in weight during the period of vitamin B_2 deprivation was, however, greater when the diet of the rats contained the unpurified caseinogen, the average being 20 g. in the first 5 weeks as against 8·5 g. on diets containing the "Lister Institute purified" caseinogen. This might be taken as an indication that, when unpurified, this caseinogen contained traces of vitamin B_2.

In this connection it may be noted that various authors have found that dermatitis occurred when the rats received some vitamin B_2 and not when complete deprivation was ensured. Sherman and Sandels [1931] obtained dermatitis in rats receiving very small doses of vitamin B_2, but Leader [1930] and Sure and Smith [1930–31] administered large doses of marmite extract or autoclaved yeast (10 % in diet) and obtained symptoms of dermatitis at the same time as good growth. These results are difficult to reconcile with those obtained here, where dermatitis has never developed in rats receiving doses of vitamin B_2, however small, and autoclaved yeast in as small quantities as 0·2 g. daily has been found to cure the symptoms [Chick and Roscoe, 1927].

In the present experiments, the use of unpurified caseinogen which may have contained small amounts of vitamin B_2 was not found to increase the incidence of dermatitis.

In addition to the above the following diets were tried, but no influence on the incidence of dermatitis was detected.

(*a*) A diet containing 17 % sucrose, with or without supplements of an alcoholic extract of marmite, containing small amounts of vitamins B_1 and B_2 [Leader, 1930].

(*b*) A diet containing traces only of fat. Evans and Lepkovsky [1929] found that dermatitis occurred more often in rats on a low fat diet.

(6) *Influence of initial reserve stores of vitamin B_2 possessed by the rat.* In observations of the incidence of dermatitis among members of the same litter, great variation has been found; frequently half of the litter succumbed and half did not. Thus it does not appear that some mothers pass on stores of the vitamin to their young while others fail to do so.

Discussion.

The present attempt to discover the factors influencing the incidence of dermatitis in vitamin B_2-deficient rats must be regarded as entirely unsuccessful. Purification of the protein of the diet, to remove all traces of vitamin B_2, was not found to increase the incidence of symptoms, nor was the presence of small amounts of vitamin B_2 in the diet. No seasonal variation in incidence was observed, and no support was obtained for the theory that the irregularity observed was connected with a hitherto unrecognised and uncontrolled dietary factor, differing from that responsible for growth. Further evidence on this last question is given in the following paper [Roscoe, 1933].

Summary.

1. Dermatitis has occurred in 108 out of the 191 rats fed on vitamin B_2-deficient diets in this laboratory during the past seven years. The average time taken for symptoms to develop was 10 weeks.

2. Time of year, weight increase and various alterations in the diet were without effect on the occurrence of symptoms.

My thanks are due to Dr H. Chick for constant advice and criticism throughout the course of this work.

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CCX. THE VITAMIN B_2 CONTENT OF VARIOUS MATERIALS COMPARED BY THEIR POWER TO PROMOTE GROWTH AND TO CURE DERMATITIS RESPECTIVELY.

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It has been suggested that the promotion of growth and the prevention or cure of dermatitis are due not to one but to two separate dietary factors, contained in the component of the vitamin B complex known as vitamin B_2 [Kuhn *et al.*, 1933].

Aykroyd and Roscoe [1929]; Chick and Copping [1930]; Chick, Copping and Roscoe [1930]; Roscoe [1930; 1931] showed, however, that all the natural foodstuffs tested which promoted growth when given as source of vitamin B_2 also cured dermatitis. Halliday [1932], on the other hand, in attempts to assay the curative power for dermatitis of various heated preparations of protein-free milk, in some cases obtained growth without amelioration of symptoms. This, however, was attributed to the severity of the dermatitis, and no evidence was obtained from her work or that of the other authors enumerated above that the growth-promoting and dermatitis-curing effects were due to different factors.

In the present paper results are given of experiments in which an attempt has been made to determine the minimum amounts of various materials which will respectively cure dermatitis and promote a given weight increase. It was thought that such a comparison might show a differing distribution of the two factors, if two such exist.

Experimental.

The rats received the diet and vitamin supplements described in the previous paper [Roscoe, 1933, 1].

For the purpose of the curative tests the severity of the dermatitis was graded into three groups as follows.

(1) *Slight inflammation* of paws or nose. Inflammation of the ears or eyelids of a not severe form occurred often and was not necessarily followed by the development of more severe symptoms; it was not therefore reckoned as dermatitis unless subsequent inflammation of other parts occurred.

(2) *Marked dermatitis* of one or more limbs, nose, ears and eyelids; the skin being red, shiny, denuded of hair and sometimes oedematous.

(3) *Severe dermatitis*, showing symptoms as in (2) only more marked, the animal in this stage being very ill.

Before testing the curative action of the materials the symptoms were allowed to proceed to the second stage of marked dermatitis. Cases of slight

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dermatitis may sometimes cure spontaneously. The following materials were tested:

(i) Watery yeast extracts.

(ii) Yeast extracts heated for 5 hours at acid reaction.

(iii) Yeast extracts heated for 1 hour at alkaline reaction.

The preparation of (i), (ii) and (iii) is described in the following paper [Roscoe, 1933, 2].

(iv) Egg-white filtrate, prepared according to the method described by Chick, Copping and Roscoe [1930].

(v) Meat, minced beef steak, dried at 37° and ground.

The results of the dermatitis-curing and growth-promoting tests are given in Table I. For the latter, the criterion was the daily dose needed to promote

Table I. *Daily doses of various materials needed by young rats when given as sole source of vitamin B_2, in order to: (1) promote 50–60 g. weight increase in 5 weeks; (2) cure the dermatitis developing in the absence of vitamin B_2.*

Material	Daily dose g.* required for 50–60 g. wt. incr. in 5 weeks (*a*)	Daily dose g.* given to cure dermatitis	No. of rats: Cured	No. of rats: Slight improvement	No. of rats: No improvement	Smallest dose on which more than ½ of the rats were cured, g. (*b*)	Ratio of growth-promoting to dermatitis-curing doses (*a*/*b*)
Yeast extract							
R. VIII	0·25	0·06	—	1	—	0·125	2·0
		0·125	2				
R. X	0·25	0·06	—	1	3	0·125	2·0
		0·125	3				
R. XII	<0·25	0·06	2	—	1	0·06	<4·0
		0·125	1				
Acid autoclaved yeast extract (120°, 5 hours)							
R. V	0·25	0·06	—	1	—	0·125	2·0
		0·125	3	—	1		
		0·25	1				
R. X	0·25	0·06	2	1	2	0·125	2·0
		0·125	3	—	1		
Alkaline autoclaved yeast extract (120°, 1 hour)							
R. VIII	1·5	0·25	1	—	1	0·5	3·0
		0·5	1				
R. X	0·5	0·125	—	1	1	0·25	2·0
		0·25	3	1			
		0·5	1				
R. XII	1·0	0·125	—	—	1	0·25	4·0
		0·25	2				
Egg-white filtrate							
R. I	10	1·25	—	—	1	2·5	4·0
		2·5	2				
Meat							
Steak, dry	0·7	0·1	—	1	3	0·2	3·5
		0·2	2	—	1		

* The doses of yeast extracts are given as the equivalents in dry yeast, those of the egg-white filtrate as equivalents of fresh egg-white.

50–60 g. weight increase in 5 weeks; for the former the smallest daily dose which resulted in cures of more than half the animals. It will be seen that the amount needed to cure dermatitis varied from one half to one quarter of that required to

support the standard rate of growth. The number of rats used for each material was admittedly small so that a variation of this magnitude was not considered significant.

The results indicated that, within the large experimental error unavoidable in this type of experiment, the dermatitis-curing and growth-promoting factors have a similar relative distribution in the materials tested, yeast extract, egg-white filtrate and meat, and that they are similarly affected by heat in acid and alkaline media, both being much more sensitive in the latter case [see also the following paper, Roscoe, 1933, 2]. It is therefore concluded that the two factors are identical.

SUMMARY.

The daily doses of yeast extract, egg-white filtrate or meat needed to cure the dermatitis associated in young rats with vitamin B_2 deficiency were shown to be proportional to the daily doses needed to promote a given weight increase. This was also true of the yeast extract after it had been heated in acid or alkaline media. Thus no support was found for the theory postulating the existence of separate dietary factors responsible respectively for preventing and curing dermatitis and for promoting growth.

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CCXI. THE HEAT-STABILITY OF VITAMIN B_2. III. THE RATE OF DESTRUCTION AT VARIOUS REACTIONS OF VITAMIN B_2 CONTAINED IN DIFFERENT MATERIALS.

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While little is known of the chemical and physical properties of vitamin B_2, it is generally recognised to be the most heat-stable member of the vitamin B complex. This fact made possible its original differentiation from vitamin B_1 and has been the basis of most subsequent methods of separation from other constituents of the vitamin B complex. For this reason much interest and work has been concentrated on ascertaining the degree of this heat-stability and the conditions governing it.

Williams and Waterman [1929] compared the effect on growth of rats of a given amount of brewer's yeast, given as source of vitamin B_2, before and after autoclaving for 6 hours at 120° at varying p_H, and showed that after this length of time destruction was slight in an acid medium but considerable at a slightly alkaline reaction (p_H 8·0) and practically complete at a strongly alkaline reaction (p_H 12–14).

Papers I and II of this series [Chick and Roscoe, 1930; Chick and Copping, 1930] contained the results of experiments on the heat-stability of vitamin B_2 as contained in dried yeast or in a watery yeast extract. The assays were carried out by means of rat growth tests and were checked by cures of dermatitis. It was shown that after heating at 120° for 4–5 hours in an acid medium (p_H 3·3–3·0) about half of this vitamin was destroyed, but after 4 hours in a slightly alkaline medium (p_H 8·3–7·1) nearly all was destroyed, while in a more alkaline medium (p_H 9·9–8·7) destruction was complete.

Halliday [1932] studied the heat-stability of vitamin B_2 present in protein-free milk, in quantitative experiments comparing the minimum amounts of each material needed to produce a given weight increase. Heating for 1 hour at 100° at p_H 4·3, 7·0 and 10·0 caused 10, 30 and 40 % loss, respectively, of vitamin B_2. Four hours' heating at the same reactions resulted in 30, 50 and 75 % loss respectively. It was therefore apparent that the rate of destruction by heat increased with increasing alkalinity and that after 4 hours at a temperature of 100° in a strongly alkaline medium there was great destruction of vitamin B_2.

In apparent contradiction of these results are those of Hassan and Drummond [1927], Reader [1929; 1930], Guha and Drummond [1929], Narayanan and Drummond [1930], Guha [1931, 1], who found that rats grew normally when receiving vitamin B_2 as small daily doses of marmite which had been heated at 110–125° for 1–3 hours in alkaline media, and who therefore concluded that vitamin B_2 was stable to heat and alkali. Only in two cases, however, was the marmite tested for its vitamin B_2 content before heating [Hassan and Drummond, 1927; Reader, 1929] and in no case was a determination made of the minimum doses of the material needed for normal growth before and after heating, so that the only conclusion to be drawn from this work is that vitamin B_2 was not entirely destroyed, in the materials tested, by 1–3 hours' heating in the alkaline medium employed.

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The apparently divergent results obtained by different workers when investigating the heat-stability of vitamin B_2 as present in different materials have led to the supposition that variations in the source of the vitamin might affect the heat-stability. This factor was stressed by Guha [1931, 2]. He investigated the effect of heat (3 hours at 124–125°) on three different materials, (1) fresh ox-liver, (2) a watery solution of commercial liver extract (Eli Lilly, No. 343) and (3) a watery extract of brewer's yeast. In acid medium, no destruction of vitamin B_2 was observed in (2) or (3). But after heating in alkaline medium (initial p_H 9·0, final 7·6–6·1) the vitamin B_2 in (1) and (3) suffered 75 % and 90 % destruction respectively, while that in (2) appeared to be unaffected. These results together with previous ones were taken to show that the vitamin B_2 present in commercial liver extract (and in marmite, see Hassan and Drummond [1927]) was not readily destroyed by heat and alkali as was the case when present in fresh liver or in a watery yeast extract, the greater stability in liver extract and marmite being probably due to the presence of some protective agent.

In this paper therefore, experiments are reported, using various sources of vitamin B_2 similar to the above, *viz.*: a watery yeast extract, a watery liver extract and marmite. These have been heated at 120° for 1 hour in alkaline solution at approximately equal p_H values. In addition, further results are given of the effect of heating yeast extracts at acid and various alkaline reactions.

Experimental.

The method of testing for vitamin B_2 was that described by Chick and Roscoe [1928]. The basal diet and vitamin B_1 and B_4 supplement employed are described in an accompanying paper [Roscoe, 1933, 1]. The young rats weighing 35–45 g. were fed the basal diet only for 1 week during which time their weight was stationary and then received the vitamin B_1 and B_4 supplement and the doses of vitamin B_2. Growth was observed for 5 weeks, the amounts of each material providing sufficient vitamin B_2 to promote 50–60 g. increase in weight in this time being ascertained. This amount of growth was subnormal, and with more vitamin B_2 much better growth occurred; there was therefore no uncertainty, such as occurs when normal growth is the criterion, as to whether the doses administered were indeed the minimum amounts required to support this degree of growth. Four rats received each dose.

The following three materials were investigated.

(1) A watery extract of yeast, made from washed brewer's yeast with boiling 0·01 % acetic acid. 2 cc. of the extract were equivalent to 1 g. of the original yeast (dry wt.).

(2) Marmite, an autolysed yeast extract. This was dissolved in an equal weight of water before heating.

(3) Liver extract (Eli Lilly, No. 343), kindly supplied by the maker in the watery form prepared for intramuscular injection. 1 cc. of the extract was equivalent to 5 g. of the original whole liver.

These materials were all heated for 1 hour at 120° at approximately equal degrees of alkalinity (p_H 8·7–7·2). In addition, tests were made of the yeast extract (1) after treatment for 1 hour at three different degrees of alkalinity and for 5 hours in an acid medium. The heating took place in an autoclave at 15 lbs. pressure. Determinations of the p_H of the materials before and after treatment were carried out by means of a hydrogen electrode.

Results.

The figures in Table I show that when watery yeast extract, marmite and liver extract were heated at 120° for 1 hour at p_H 8·7–7·2, 50 % destruction of the vitamin B_2 took place in each case. Thus there was no significant difference in the degrees of destruction by heat under these conditions of the vitamin B_2 contained in the three different materials.

Table I. *Destruction by heat of vitamin B_2 as contained in different materials.*

The following data are shown.

(*a*) The doses in g. of each material found necessary to promote standard growth (50–60 g. weight increase in 5 weeks), or, where this was not found, the nearest dose tested.

(*b*) The average weight increase in g. of the four rats receiving (*a*).

(*c*) The percentage destruction of vitamin B_2, calculated from (*a*).

	Untreated		After heating 1 hour at 120°			
Material	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	p_H during heating
Yeast extract, R. X	0·25	(69)	0·5	(57)	50 % +	8·7–7·2
Marmite	0·15	(54)	0·3	(51)	50 %	8·6–8·5
Liver extract, Eli Lilly, No. 343	0·075	(49)	0·15	(48)	50 %	8·7–7·7

In Table II are shown the results of a more extensive set of experiments on the heat-stability of the vitamin B_2 in the yeast extract. The results of some previous work with similar extracts [Chick and Roscoe, 1930] are included for comparison.

Table II. *The effect of variation in the p_H of the substrate, and in time of heating, on the destruction of vitamin B_2 contained in a watery yeast extract.*

The following data are shown.

(*a*) The doses in g. of each material found necessary to promote standard growth (50–60 g. weight increase in 5 weeks), or, where this was not found, the nearest dose tested.

(*b*) The average weight increase in g. of the four rats receiving (*a*).

(*c*) The percentage destruction of vitamin B_2, calculated from (*a*).

	Time during which material was heated at 120°								
	Untreated		1 hour			4–5 hours			
Yeast extract no.	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	p_H during heating
R. X	0·25	(69)	—	—	—	0·25	(60) (5 hours)	0 % +	1·4
XII	0·20	(56)	—	—	—	0·4	(60) (4 hours)*	50 %	3·3–3·0
XII	0·20	(56)	—	—	—	Trace undestroyed (4 hours)*			8·3–7·1
R. X	0·25	(69)	0·5	(57)	50 % +	—	—	—	8·7–7·2
R. VIII	0·25	(58)	1·5	(53)	83 %	—	—	—	9·0–8·7
R. XII	0·25	(78)	1·0	(60)	75 % +	—	—	—	9·3–8·5
XII	0·20	(56)	—	—	—	Complete destruction (4 hours)*			9·9–8·7

* Results obtained by Chick and Roscoe [1930].

As would be expected, the length of time for which heating was carried on had a considerable effect on the amount of destruction. Thus, while heating at 120° at p_H 8·7–7·2 for 1 hour destroyed only slightly more than 50 % of the vitamin, after 4 hours only traces remained undestroyed; in a more alkaline medium (p_H 9·3–8·5) after 1 hour rather more than 75 % destruction had occurred and after 4 hours (p_H 9·9–8·7) destruction was complete.

The rate of destruction was increased greatly with increasing p_H. While at p_H 1·4 an insignificant amount of the vitamin was destroyed even after 5 hours at 120°, at p_H 3·3–3·0 there was 50 % destruction after 4 hours; at a slightly alkaline reaction, p_H 8·7–7·2, the same percentage destruction occurred after 1 hour's heating, and at p_H 9·0–8·7 there was 83 % destruction after 1 hour.

Some of the above materials were also tested, before and after treatment, for their capacity to cure dermatitis [see Roscoe, 1933, 2]. The results were in good accordance with those obtained by the growth method.

Discussion.

The results here obtained on the heat- and alkali-stability of vitamin B_2 in marmite and liver extracts are at variance with those of Hassan and Drummond [1927] and Guha [1931, 2], who found no appreciable destruction of vitamin B_2 in these materials after 1 or 3 hours' heating in an alkaline medium at 115°, as opposed to considerable destruction in the case of a watery yeast extract. In the present work the vitamin B_2 in marmite and in liver extract was as much affected as that in yeast extract.

The treatment here employed was similar to, but not identical with, that used by the above authors. The marmite was heated in a slightly, as opposed to a strongly alkaline medium [Hassan and Drummond, 1927]; the liver extract was heated at a similar reaction in both instances, but in this work for 1, as opposed to 3, hours [Guha, 1931, 2]. The treatment in the present work was thus in each case less severe, so that less, rather than more, destruction was to have been expected. Hassan and Drummond, however, did not determine the minimum amounts of the untreated marmite needed for normal growth, so that their results, while showing that adequate vitamin B_2 to support growth had survived the treatment, did not prove that some degree of destruction had not occurred. The data in Guha's paper are not given in sufficient detail to show whether this criticism may not be applied to his experiments also.

The results reported in this paper emphasise the fact that the destruction of vitamin B_2 by heat should be regarded as a time-process. As a result of observations on heating for 1 hour [Hassan and Drummond, 1927; Reader, 1929], the vitamin was judged to be very heat-stable in an alkaline medium, whereas Chick and Roscoe [1930], from experiments in which heating was carried out for 5 hours, considered that the vitamin was heat-labile in an alkaline medium. To a certain extent this discrepancy is explained when the different durations of heating are taken into account.

Summary.

1. No difference was found in the stability to heat and weak alkali of vitamin B_2 as contained in (*a*) a watery yeast extract, (*b*) marmite solution or (*c*) a watery liver extract (Eli Lilly, No. 343). When heated for 1 hour at 120° at p_H 8·7–7·2 all three materials lost 50 % of their original vitamin B_2 potency. Thus no support was obtained for the theory that the resistance of vitamin B_2 to heat and alkali varies according to the material in which it is present.

2. In confirmation of previous work vitamin B_2 was found to be relatively heat-stable in acid solution and the rate of destruction to be increased rapidly with increasing alkalinity.

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CCXII. THE MICRO-DETERMINATION OF BROMINE IN BLOOD.

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For certain biochemical work it is necessary to determine the bromine content of blood in circumstances which preclude the use of samples of greater volume than 1 or 2 cc.

Experience gained during an investigation carried out for the Medical Research Council on methods for the determination of iodine in blood suggested that a method of destroying organic matter involving ignition with alkali under controlled conditions might prove to be satisfactory, and that the bromine might subsequently be determined by a micro-modification of the chromic acid aeration process [Baughman and Skinner, 1919; Evans, 1931].

In the chromic acid aeration processes described by these workers, a double aeration is necessary when chlorides are present, to eliminate errors due to a small amount of chlorine evolved during the first aeration.

In attempting to evolve a micro-method of aeration, a considerable amount of work has been done, the earlier results being promising but erratic at times. When a double aeration was employed, the recovery of bromine varied, and was sometimes as low as 60 %.

As the amounts of bromine to be determined are small (about 0·02 mg.) some variation in the recovery is to be expected, but it appeared that, with a method employing double aeration, the losses would be higher than desirable.

In the later experiments a chromic-phosphoric acid mixture was used for liberating the bromine, and it was found that the conditions could be so adjusted that satisfactory figures were obtained by one aeration in the presence of an amount of sodium chloride approximating to that present in 2 cc. of blood (Table I). The recovery of bromine is within ± 10 % of the theoretical, which is satisfactory for work of this kind.

Table I. *Determination of potassium bromide added to sodium chloride.*

	Bromine added mg.	Bromine found mg.
Control, 20 mg. of bromide-free NaCl	Nil	Nil
Ditto *plus* 0·005 mg. of bromine (KBr)	0·005	0·0049
,, 0·010 ,,	0·010	0·0099
,, 0·020 ,,	0·020	0·0214
,, 0·030 ,,	0·030	0·0327
,, 0·050 ,,	0·050	0·0515

Preparation of reagents.

Distilled water. The water should be treated with a mixture of potassium carbonate and potassium permanganate and distilled to remove bromine compounds and organic matter.

N potassium hydroxide. Prepared from specially selected material or freed from bromine by electrolysis.

20 % solution of sucrose. This solution is added to ensure that in the "blank" determinations, any bromate present is reduced to bromide. In the actual blood determinations, any bromate will be reduced during the incineration of the blood. The sucrose solution should be substantially free from bromine compounds, but it is added to the blood determinations as well as to the "blanks" so that any correction necessary for bromine present in the sucrose is made automatically.

Chromic-phosphoric acid mixture. (1) Stock solution. Pure syrupy phosphoric acid, sp. gr. 1·75, is treated with sufficient chromic acid to produce a saturated solution and leave an excess of chromic acid undissolved, when the mixture is heated in a boiling water-bath for 3 hours, with occasional stirring. This procedure also destroys organic matter and decomposes any chloride or bromide.

The mixture is allowed to cool overnight, and is then freed from excess of chromic acid by filtration through glass wool. The solution is now again heated in a boiling water-bath and is aerated for 3 hours with a vigorous stream of air to remove chlorine or bromine. The air should be filtered through a plug of cotton wool to remove dust and then through a plug of glass wool to remove cotton wool fibres. After cooling, the solution is ready for dilution with the special distilled water.

(2) Diluted solution. Before being used under the conditions of aeration to be described later, the stock chromic-phosphoric solution must be diluted slightly so that, with 20 mg. of bromide-free sodium chloride, under the standard conditions of aeration, no iodine is liberated in the potassium iodide bubblers, and the recovery of bromine from potassium bromide in the presence of the sodium chloride is satisfactory (see Table I). Dilution of 90 cc. of the stock solution with 5·0 cc. of water usually provides a solution of the required concentration, but it is necessary to examine every batch of stock solution to ascertain whether it functions properly when so diluted.

2 % aqueous solution of potassium dichromate.

2 % solution of potassium iodide. This solution should be prepared from distilled water freed from dissolved gases by recent boiling. When protected from the action of daylight and oxidising vapours, the solution is quite stable, and a fresh solution need only be prepared every 2 or 3 weeks.

0·5 % solution of soluble arrowroot starch. This solution, which must be freshly prepared, is obtained by rubbing up the starch with a little water, and pouring the mixture into boiling water. The total time of boiling should not exceed 2 minutes.

Standard N/500 solution of sodium thiosulphate. This solution is made with distilled water freed from dissolved gases by boiling and containing 1 % by volume of pure amyl alcohol, as recommended by Aitken [1930]. It should be stored in a container fitted with a soda lime tube and a siphon made entirely of glass terminating in a glass tap. When shielded from direct daylight, the solution is quite stable, but should be restandardised at least once a week. The solution may be standardised against a solution of potassium bi-iodate, $N/100$ in oxidising power.

Destruction of the organic matter.

1–2 cc. of blood, carefully measured or weighed, are placed in a round-bottomed nickel dish, 9 cm. in diameter, and treated with 20 cc. of water, 3·0 cc. of N KOH, and 0·1 cc. of the sucrose solution. The mixture is well stirred with a glass rod, the rod being rinsed with a little water and removed.

After taking the solution to dryness on the steam-bath, the dish and its contents are placed in an oven at 150–160° for 1 hour and then ignited for 1 hour in a muffle furnace at 480–500°.

The material is now moistened with about 20 cc. of water, the carbon is thoroughly broken up with a glass rod, and the mixture is allowed to digest on the steam-bath for 2–3 minutes while being stirred with the rod.

The hot solution is now decanted through a filter (9 cm. Whatman No. 5 paper previously thoroughly washed with hot distilled water before use) into a 100 cc. beaker, the carbon in the dish being again extracted by digestion with water on the steam-bath and finally extracted with water until the filtrate has a volume of about 40 cc. The filtrate is evaporated on the steam-bath in a recently ignited platinum dish, about 8 cm. in diameter.

The filter-paper is placed in the nickel dish, and the contents of the dish are moistened with water and treated with 1·0 cc. of *N* KOH. After being mixed thoroughly, the mixture is evaporated to dryness on the steam-bath, dried for 15 minutes at 150–160° and ignited in the muffle furnace at 480–500° for 1 hour.

The residue, which now contains only a little carbon, is extracted by digestion with water on the steam-bath in the manner already described, the filtrate being added to the previous filtrate which has been evaporating in the platinum dish.

(*Note*. The nickel dishes should be freed from carbon after use by igniting them in the muffle furnace.)

The combined filtrates in the platinum dish are evaporated to dryness, the dish is placed in the oven at 150–160° for 10–15 minutes and is then ignited for 10 minutes in the muffle at 480–500°.

The residue is dissolved in a little water, the solution is evaporated, and the dish is placed in the oven at 150–160° for 10–15 minutes and ignited for 10 minutes in the furnace at 480–500°.

The moistening, evaporation, drying and ignition for 10 minutes are repeated once again.

To assist the final removal of traces of organic matter, the residue is now dissolved in a little water, treated with 1 cc. of 2 % potassium dichromate solution, and the evaporation, drying and ignition for 10 minutes at 480–500° are repeated.

The alkaline residue is dissolved in about 15 cc. of cold water and the solution is passed through a filter (7 cm. Whatman No. 42, previously well washed with hot water), washing with cold water until the filtrate has a volume of 30–40 cc.

The filtrate is evaporated in the platinum dish; the dish is placed in the oven at 150–160° for 10–15 minutes and is finally ignited for 5 minutes at 480–500°.

The dish is now covered to prevent contamination by organic matter in the form of dust from the atmosphere.

Aeration and titration of the liberated iodine.

The apparatus (Fig. 1) must be kept scrupulously clean and free from minute traces of organic matter. On no account must lubricating grease or rubber joints be used, the ground joints being lubricated with distilled water only.

Frequent cleansing by soaking in chromic-sulphuric cleaning mixture is essential, and this should be followed by very thorough washing, first with tap water, and then with distilled water. Finally, the entire apparatus, with the exception of the two small potassium iodide-starch containers, is rinsed with

very dilute sulphuric acid (approx. $N/5000$), well drained and set up in a position where it is screened from the action of direct sunlight.

(*Note.* The action of daylight encourages the reaction,

$$2Br_2 + 2H_2O = 4HBr + O_2,$$

which reaction, should it take place in the tubes D and E, will prevent the bromine from being completely evolved. Similarly, if the tubes are not kept slightly acid, but tend to be alkaline, evolution of the bromine will be incomplete.)

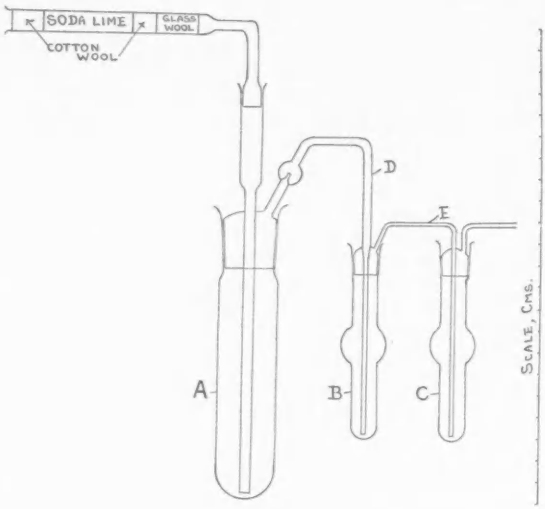

Fig. 1.

Each of the small bubblers B and C contains 0·5 cc. of the potassium iodide solution and 0·5 cc. of the starch solution[1].

The residue in the platinum dish is dissolved in exactly 3·0 cc. of water, and the solution is carefully poured down the funnel into the large bubbler A, the dish being twice rinsed with exactly 1·0 cc. of water, the total volume of water used being 5·0 cc.

The water-pump is now turned on, and a slow stream of air at the rate of about 3 litres per hour[2] is passed through the apparatus, while 5·0 cc. of the diluted chromic-phosphoric mixture are carefully run down the funnel into the large bubbler. The heat of reaction causes the temperature of the solution to rise slightly.

The soda-lime tube is replaced, and, in about 15 secs. when the effervescence ceases, the rate of the air stream is increased to 8–10 litres per hour and maintained at this rate for 20 minutes (laboratory temperature 15–20°).

The contents of the second small bubbler C, which should contain little or no liberated iodine, are now blown back into B, and the end of the tube D is

[1] The addition of starch to the bubblers is very beneficial in assisting the retention of the liberated iodine [Thomas and Cross, 1928; Zepf and Vetter, 1930].

[2] If the air stream is too rapid, small drops of the alkaline solution may be splashed into the upper part of the large bubbler and may absorb some of the evolved bromine.

rinsed with a little water, whereby the total volume of liquid in the container of bubbler *B* is brought up to 3–4 cc.

The iodine is now titrated with *N*/500 thiosulphate, using a micro-burette. In the work for the Medical Research Council previously mentioned it was found necessary to make a small correction for the end-point error. In terms of bromine, for small volumes, this correction is $+0{\cdot}0002\ V$ mg., where V = the volume of the solution after titration (*i.e.* one adds to the figure for bromine obtained by titration, 0·0002 mg. for every cc. of solution).

Some results obtained by the method are given in Table II. These form a complete series of results and are not selected figures.

Table II.

Recovery of bromine compounds added to 2·0 cc. of horse blood in each case.

Results obtained by deducting the figures found for the bromine naturally present in the blood.

Form in which the bromine was added to the blood	mg. of bromine added	mg. of bromine found
Potassium bromide	0·010	0·011
,,	0·020	0·022
,,	0·030	0·030
o-Bromobenzoic acid	0·010	0·012
,,	0·020	0·019
,,	0·016	0·017
,,	0·024	0·027
,,	0·030	0·031
,,	0·024	0·027
,,	0·030	0·031
,,	0·040	0·040
Bromodiethylacetylurea	0·010	0·010
,,	0·016	0·015
,,	0·026	0·026
,,	0·030	0·030

Bromine found in samples of blood kindly supplied by Dr J. B. Orr.

		Total mg. of bromine found	mg. of bromine per 2·0 cc. of blood corrected for "blank"
"Blank"		0·0007	—
,,		0·0009	—
*1. Cow	0·98 cc.	0·0171	0·033
,,	1·94 ,,	0·0349	0·035
2. Guinea-pig (F).	0·97 ,,	0·0217	0·043
,,	1·92 ,,	0·0492	0·050
3. Fowl (M).	0·98 ,,	0·0075	0·014
,,	1·94 ,,	0·0134	0·013
4. Sheep (F.)	0·94 ,,	0·0092	0·018
,,	1·86 ,,	0·0171	0·018
5. Rat (M).	0·93 ,,	0·0116	0·023
,,	1·84 ,,	0·0198	0·021

* This animal had been receiving a diet containing potassium iodide. The figure obtained for bromine may therefore be slightly higher than the true value.

Frequent "blanks" should be carried out to ensure that bromine is not being picked up from the atmosphere of the laboratory or other sources. If the blood is oxalated, then the "blanks" must contain a corresponding amount of the oxalate.

Satisfactory results will only be obtained when strict attention is paid to details. The aerations must be carried out in precisely the manner described, and constant vigilance must be exercised to ensure that errors are not creeping in from unexpected sources. It appears to be desirable to call attention to the following points.

1. The presence of minute traces of organic matter in the apparatus may cause low results as also may the presence of foreign organic matter in the starch solution.

2. If the potassium iodide or starch solutions are alkaline, the results will be low.

3. It is not permissible to use chromic-phosphoric mixture which evolves a small amount of halogen on aeration and to correct for this halogen by means of a "blank." Such a "blank" may be due to elemental chlorine in the chromic-phosphoric solution, which, while being evolved in the "blank," may not be evolved in the actual determination owing to its interaction with the bromide present.

4. High results may be obtained with materials containing iodine. Normally, blood does not contain sufficient iodine to affect the bromine figures.

5. For the recovery of volatile bromine compounds, such as bromoform, the blood would have to be evaporated and burnt in a closed system. The figures obtained (Table II) indicate that the method of alkaline ignition in open dishes is satisfactory with bromine compounds of the nature of those likely to be present in blood.

6. If colloidal silica were present in the large bubbler during aeration, the removal of the bromine would probably be incomplete. Such silica might be derived from the caustic potash or from the material undergoing examination, but no trouble of this kind has been experienced while working with the samples of blood.

7. Examination of the results embodied in Tables I and II shows that, although no chlorine is evolved when working with pure sodium chloride, the figures for bromine tend to be high rather than low, although it was found that, in the absence of chloride, the chromic-phosphoric aeration process always gave low results for the recovery of bromine (about 85 % recovery). As this error tends to increase with increased bromine concentration it appears that, when carrying out a series of comparative determinations of blood-bromine, the results will be more strictly comparable if the volumes of blood taken for analysis are such that approximately the same quantity of bromine is recovered in each case.

SUMMARY.

A method is described for the determination of bromine in small volumes of blood with an accuracy of approximately 10 %.

In conclusion, we wish to thank Sir Robert Robertson for granting us permission to publish this paper, and Dr J. B. Orr for arranging for the supply of samples of blood.

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CCXIII. THE PRODUCTION OF MUCUS DURING THE DECOMPOSITION OF PLANT MATERIALS[1].

I. THE EFFECT OF ENVIRONMENTAL CONDITIONS.

By JAGESHWAR GOPAL SHRIKHANDE.

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(*Received July 27th, 1933.*)

It has been observed that the straw in some manure heaps undergoing decomposition develops stickiness. Such sticky manures may be better adapted to light sandy soils and are possibly unsuitable for heavy clays. The possible effect of mucilage from plant residues on the physical behaviour of the soil has been suggested by Hutchinson and Clayton [1919]. The substance conferring on manures the physical property of stickiness will be referred to hereafter as mucus. It is apparent that mucus formation must be a result of the microbiological activity during decomposition.

Though the decomposition of plant materials has been studied extensively and the losses of individual constituents followed in detail by Rege [1927], Waksman [1928] and Norman [1929], no reference is made in the literature to the production of mucus.

The experiments described in this paper were designed to examine the conditions involved in the production of mucus. Straw was fermented in the presence of different sources of available nitrogen with changes in the physical conditions and reaction. Extractions of the decomposed straws were made to seek any possible correlation between the rates of their decomposition and the production of mucus. The amount of mucus produced was measured by a specially devised physical test.

Physical test for measuring the stickiness.

The principle of the method consists in measuring the vertical force required to separate after drying two metal plates which contain between them a known weight of the manure sample.

The following apparatus is required for the determination—a number of pairs of metal plates, one of 4 ins. square and the other of 3 ins. square with a hook in the centre, a system of two frictionless pulleys in a horizontal plane, lead shot, a receptacle to receive the lead shot and a lead weight weighing 1600 g. with a slit to fit over the hook of this plate. This apparatus is represented diagrammatically in Fig. 1.

The metal plates should be sufficiently rigid not to bend under the forces applied. In order to keep the lower plate firmly fixed, while the force is being

[1] This paper is an abridged form of a part of the thesis approved for the Degree of Doctor of Philosophy in the University of London.

applied, it is made to slide in between two jaws made by screwing to the bench top two metal pieces over another of 4 ins. length and of the same thickness as that of the plates. The lower two plates are kept parallel and 4 ins. apart in such a way that the lower metal plates of 4 ins. square can be easily slid in with no danger of causing any jerks to the plates containing the manure. When the plates are ready for measuring the pull, the lower plate is gently slid in, the loop at one end of the string over the pulleys is put in the hook of the top plate and

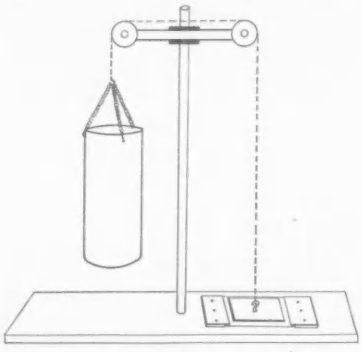

Fig. 1. Apparatus used for measuring stickiness.

the other is connected with the receptacle for carrying the lead shots. The shots are gradually poured in till the top plate is vertically lifted instantaneously from its position of rest. The weight of the shots *minus* the weight of the top plate gives the total pull to separate the two plates. The diameter of the circular block occupied by the manure is measured. An average of three or four readings in different positions is taken as the block is not usually regular. Knowing the diameter and the pull recorded, the force per unit area can be calculated.

The procedure consists in cutting the rotted straw as finely as possible, after which the mass is thoroughly mixed and vigorously worked with a spatula to obtain an even mixture. One gram portions are then weighed out on each of the plates. The mass is gathered into a mound by the spatula in the centre of the plate and the top plate placed in such a way that it rests freely with faces parallel. It is then pressed with the lead weight for 15 seconds and dried at 100° in an oven overnight to ensure uniform and complete drying. It is essential in all the experiments to keep constant the weight and time for pressing the plates, because the area of the manure and its height or thickness depend upon the force with which the top plate is pressed against the manure surface, whilst the pull required to separate the two plates depends upon the distance between them and therefore upon the thickness of the manure disc. In his early experiments on the stickiness of soils Kachinski [1930] left a weight on the sample for a specified time. Bouyoucos [1932] however pressed the plates with the hands, introducing different pressures at different times, without considering the thickness of the disc occupied by the soil.

Table I gives the significance of the determinations of stickiness made by the physical test. It will be seen that the magnitude of the error increases inversely with stickiness and that with highly sticky and moderately sticky samples the figures for stickiness are significant.

Table I. *Significance of the determinations of stickiness made by the physical test.*

Readings	Actual pull recorded in g.	Radius of the manure disc in cm.	Force per unit area per 1 g. of wet sample	Force per unit area per 1 g. of dry sample
	$NaNO_3$ rot: dry matter 13 %. Very sticky.			
1	5200	1·25	1059	8140
2	4875	1·25	991	7620
3	4958	1·25	1010	7770
4	4800	1·20	1061	8160
5	4110	1·25	837	6436
6	5190	1·25	1056	8120
7	5000	1·25	1018	7830
	5075	1·25	1034	7944
		Mean		7752·5
		Standard deviation		566·32
		Standard error of the mean		± 200·2
	Urea rot: dry matter 20 %. Moderately sticky.			
1	2000	1·30	481·4	2407
2	1800	1·25	366·4	1832
3	1200	1·25	244·5	1222
4	1600	1·20	353·7	1768
5	1900	1·20	420·1	2100
6	2000	1·25	407·3	2036
7	2050	1·25	417·4	2087
8	1800	1·20	390·0	1990
		Mean		1930·2
		Standard deviation		345·0
		Standard error of the mean		±121·9
	$(NH_4)_2CO_3$ rot: dry matter 12 %. Slightly sticky.			
1	130	1·80	12·7	105·8
2	150	1·75	13·5	101·2
3	100	1·40	16·2	135·3
4	150	1·70	16·5	137·7
5	290	1·60	36·0	300·5
6	100	1·60	12·4	103·5
7	250	1·50	35·3	294·1
8	220	1·60	27·3	227·5
		Mean		175·7
		Standard deviation		85·4
		Standard error of the mean		±30·2

Experimental.

The physical test for stickiness was carried out on decomposed straws obtained under the various conditions outlined below.

Oat straw was rotted in presence of mixed flora for 30 days with the following changes in the environmental conditions.

(*a*) Variation in the initial moisture content at 35°.

(*b*) Variation in the sources of nitrogen at 35°.

(*c*) Variation in temperature—15°, 25°, 35° and 45°—with urea as the source of nitrogen.

(*d*) Variation in temperature—15°, 25°, 35° and 45°—with sodium nitrate as the source of nitrogen.

(*e*) Degree of decomposition at 35° with sodium nitrate as the source of nitrogen.

(*f*) Adjustment of p_H at 10·0 independent of the source of nitrogen.

Technique and methods.

Twenty g. of air-dry chaffed oat straw of known moisture and nitrogen contents were fermented aerobically in bottles with its natural mixed flora. Nitrogen was supplied to the extent of 1 g. per 100 g. of straw and the bottles incubated at the desired temperature. They were turned round in the first few days to ensure thorough wetting and frequently stirred to get homogeneous distribution of nitrogen and moisture. Water-logging was avoided as it causes anaerobic conditions. After the desired period each bottle was weighed with its contents and analysed as indicated below.

1. Extractions with water, 1 % sodium carbonate and 1 % sodium hydroxide. In each case 10 g. of the wet sample were boiled for 5 minutes with 100 cc. of water and filtered. The aqueous extract was evaporated to dryness on a water-bath and weighed. The alkaline extracts were precipitated with a few drops of hydrochloric acid, gently heated to coagulate the precipitate and then filtered through tared papers. The precipitates were well washed, dried and weighed.

2. Extractions with 90 % alcohol were carried out in a Soxhlet apparatus for 6 hours. Upon removal of the alcohol the extract was weighed, and after hydrolysis by boiling with 5 % H_2SO_4 for 2 hours its sugar content was determined by the Mohr-Bertrand method.

3. Treatment with hydrogen peroxide: the method employed was described by Shrikhande [1933].

4. Ammonia-N was determined by distillation with MgO.

5. Nitrate-N on the samples rotted with sodium nitrate was determined by distillation of the residue from ammonia-N in presence of alkali and Devarda's alloy.

6. Total N was determined by the usual Kjeldahl method. In the presence of nitrate-N the sulphuric-salicylic acid method was adopted.

Results.

Series (a) and (b). Fungi were noticed on or about the 6th day. At the end of 30 days no trace of fungus mycelium was obvious except with ammonium carbonate and urea. *Coprinus* seemed to be very active in ammonium carbonate, urea and sodium nitrate rots. Better decomposition was observed with ammonium sulphate when calcium carbonate was introduced. Fungus growth was hardly noticeable at 15°. The rots appeared different under different conditions. The manures obtained, using ammonium carbonate, sodium nitrate and mould tissues as the source of nitrogen at high moisture contents, were noticeably slimy.

Effect of initial moisture content.

Table II gives the effect of initial moisture content upon the decomposition and nitrogen transformation. In each case the moisture content initially was adjusted at 60, 70, 80 and 90 %. During the course of decomposition an attempt was made to maintain the above levels of moisture but at the end of 30 days they seem to narrow down to about 85 to 98 %, which seems to be the optimum moisture necessary for pronounced rotting.

The series with ammonium carbonate, ammonium sulphate and sodium nitrate were repeated twice. Series (*a*) was used for nitrogen determinations and series (*b*) for extractions and the physical test for stickiness. The figures for moisture content and the losses of dry matter indicate that the higher the moisture content the greater the activity of the organisms and consequently the greater is the degree of decomposition. This observation agrees with that of Engberding [1909] who found an increased number of bacteria with the increased

Table II. *Nitrogen content of straws rotted by mixed floras with different sources of available nitrogen for 30 days at 35°.*

Expressed on 100 g. dry straw.

Initial moisture %	Dry matter	Loss of D.M.	NH_3-N	NO_3-N	Total N	N factor	N equiv.
			Ammonium carbonate.				
60	(*a*) 13·8	39·6	0·018	0·0	1·20	0·89	2·2
	(*b*) 19·7	36·5					
70	(*a*) 12·8	40·6	0·016	—	1·33	0·96	2·3
	(*b*) 15·0	39·2					
80	(*a*) 12·0	41·2	0·013	—	1·33	1·06	2·5
	(*b*) 14·0	45·0					
90	(*a*) 11·9	42·2	0·044	—	1·71	1·20	2·8
	(*b*) 11·4	44·4					
			Ammonium sulphate.				
60	(*a*) 17·0	36·5	0·467	—	1·29	0·68	1·8
	(*b*) 23·4	30·1					
70	(*a*) 15·9	28·5	0·595	—	1·49	0·59	2·1
	(*b*) 19·3	32·5					
80	(*a*) 15·4	23·4	0·550	—	1·41	0·69	3·0
	(*b*) 16·7	31·5					
90	(*a*) 14·0	20·0	0·593	—	1·68	0·68	3·4
	(*b*) 14·7	30·7					
			Sodium nitrate.				
60	13·8	30·7	0·041	0·119	1·76	0·83	2·7
70	(*a*) 13·6	49·2	0·012	0·020	1·18	0·81	1·6
	(*b*) 15·2	52·8					
80	(*a*) 13·4	43·7	0·009	0·009	1·20	0·63	1·4
	(*b*) 13·3	52·3					
90	12·6	48·7	0·027	0·027	1·31	0·89	1·8
			Water.				
80	18·2	30·0	—	—	0·36	0·16	5·3
			Peptone.				
75	16·5	39·5	0·004	—	1·48	1·12	2·8
75	15·6	44·1	0·007	—	1·49	1·10	2·4
			Caseinogen.				
75	17·0	43·0	0·002	—	1·46	1·10	2·5
75	17·1	41·8	0·002	—	1·30	1·07	2·5
			Urea.				
75	20·0	32·5	0·003	—	1·36	1·03	3·1
75	18·9	34·0	0·004	—	1·67	1·30	3·8

moisture content of the soil. There is, however, an exception to this in the case of ammonium sulphate where the order is reversed. It is possible therefore that a high moisture content with $(NH_4)_2SO_4$ as the source of nitrogen depresses the activity of the organisms.

The figures for "nitrogen factor" of straws rotted at 35° were determined for the sake of comparison and are given in column 7 of Table II. The nitrogen factor in these cases is the resultant of the many organisms involved and shows a tendency to increase with higher moisture content, meaning that it increases with the degree of decomposition for the particular source of nitrogen. Ammonium sulphate gives the lowest nitrogen factor whereas ammonium carbonate gives the highest with inorganic sources of nitrogen. Sodium nitrate

however does not appear so good as ammonium carbonate from the point of view of nitrogen immobilisation although it gives a higher decomposition. The nitrogen factor with organic sources of nitrogen is of little value because it is difficult to distinguish between nitrogen synthesised by the organisms and the organic nitrogen supplied.

"Nitrogen equivalents" are given in Table II, column 8. Sodium nitrate gives the lowest figure of 1·4 with 80 % initial moisture content indicating the greatest activity of the organisms per g. of N. The highest figure is 3·4 with $(NH_4)_2SO_4$ and 80 % moisture, no doubt due to the low activity of the organisms in this case.

Hutchinson and Clayton [1919], while discussing the decomposition of cellulose with *Spirochaete cytophaga*, say "from the chemical standpoint and on account of its insolubility in acids and solubility in ammonium hydroxide, the mucilage would without doubt appear in the 'crude humus' fraction in the conventional soil analysis." An attempt was made to extract this fraction possibly responsible for the stickiness. Table III gives the figures for various extracts and the values for the stickiness as measured by the physical test. The figures for stickiness were obtained on the mixture of samples with the different moisture contents for a particular source of nitrogen. The maximum stickiness was obtained with sodium nitrate, which also gave the greatest decomposition and the highest water, sodium carbonate and sodium hydroxide extracts. Mould tissue is the source of nitrogen which gives the maximum decomposition. 2 % nitrogen supplied as tissue gives extracts of the same order as sodium nitrate though the stickiness is rather lower. Double the amount of tissue produces more than double the amount of stickiness. This clearly indicates that stickiness depends a great deal upon the quantity of elaborated fungal tissue. The lowest figures for stickiness and extracts were obtained with ammonium sulphate. The best correlation between stickiness and extracts seems to be obtained with sodium carbonate extraction. This is expressed graphically in Fig. 2. The

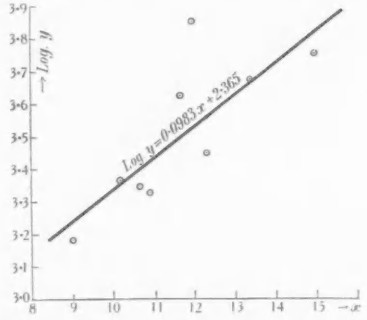

Fig. 2. Correlation between stickiness and sodium carbonate extracts of manures.

logarithm of stickiness when plotted against the extract can approximately be represented by a straight line. Log $y = 0{\cdot}0983x + 2{\cdot}365$.

The aqueous extract increases with the degree of decomposition. Water would extract some protein, very little of the hemicelluloses and any water-soluble material, gummy or otherwise, synthesised during decomposition. The larger water extract can therefore be explained by the presence of more of the

Table III. *Different extracts with physical test for stickiness and* p_H *values of straws rotted by mixed flora with different sources of nitrogen for 30 days at 35°.*

Expressed on 100 g. dry manure.

Initial moisture %	Loss of D.M.	Alcoholic extract	Sugars in alcoholic extract mg.	H_2O extract	NaOH extract	Na_2CO_3 extract	Physical test g.	Initial p_H	Final p_H	H_2O_2 extract
				$(NH_4)_2CO_3$.						
60	(*a*) 37·16	5·16	1139·5	—	—	—				
	(*b*) 36·50	6·20	—	21·1	16·9	11·5				
70	(*a*) 40·60	5·04	326·9	—	—	—				
	(*b*) 39·20	8·50	—	21·2	18·2	11·9	4241	9·0	8·0	20·2
80	(*a*) 41·20	4·30	462·5	—	—	—				
	(*b*) 45·00	5·20	—	22·2	11·4	8·8				
90	(*a*) 42·20	4·58	382·2	—	—	—				
	(*b*) 44·40	10·30	—	29·3	13·5	3·5				
				$(NH_4)_2SO_4$.						
60	(*a*) 36·50	4·19	418·0	—	—	—				
	(*b*) 30·10	9·00	—	17·9	16·8	7·7				
70	(*a*) 38·50	4·45	529·0	—	—	—				
	(*b*) 32·50	8·20	—	20·5	13·3	4·1				
80	(*a*) 23·40	4·50	520·0	—	—	—	220	5·5	6·5	14·5
	(*b*) 31·50	9·20	—	20·8	14·4	3·3				
90	(*a*) 20·0	5·30	513·0	—	—	—				
	(*b*) 30·7	9·30	—	19·8	12·2	4·5				
				$NaNO_3$.						
60	30·7	5·50	247·6	—	—	—				
70	(*a*) 49·2	3·80	104·8	—	—	—				
	(*b*) 52·8	9·10	—	44·3	17·4	15·5	7164	6·35	10·0	27·4
80	(*a*) 43·7	4·30	109·8	—	—	—				
	(*b*) 52·3	10·30	—	62·1	19·3	8·3				
90	48·7	4·35	182·0	—	—	—				
				Water.						
80	30·0	6·35	—	12·9	10·5	1·2	0	5·50	—	16·3
				Peptone.						
75	39·5	5·25	—	21·5	15·2	10·9	1504	5·75	7·5	16·3
75	44·1	7·11	—	26·9	16·6	7·2				
				Caseinogen.						
75	43·0	7·10	—	28·7	24·5	11·9	2727	—	7·5	15·8
75	41·8	11·05	—	27·2	19·4	13·5				
				Urea.						
75	32·5	8·36	—	25·3	19·7	11·6	2278	6·45	7·5	12·8
75	34·0	10·04	—	19·0	8·7	8·7				
				$(NH_4)_2SO_4+CaCO_3$.						
80	35·9	—	—	—	—	10·6	2176	—	7·5	—
				$Ca(CN)_2$.						
80	29·1	—	—	—	—	13·2	4635	—	—	—
				Mould tissue to give 1 % nitrogen.						
80	57·7	—	—	28·4	10·3	17·9	2124	—	8·2	—
				Mould tissue to give 2 % nitrogen.						
80	61·6	—	—	36·9	10·0	20·3	5622	—	8·2	—

protein and other microbial constituents which are progressively synthesised and possibly also of more gummy material which seems to increase, as indicated by the high stickiness with the physical test.

The alkaline extracts diminish with the time of decomposition. Alkaline treatment extracts more than water alone, but only a part of the substances in solution is precipitated by acid. The materials extracted with alkali are

practically of a similar nature to those obtained with hot water. Extraction may vary with increasing concentration and nature of the alkali. 1 % sodium hydroxide would extract lignin to an appreciable extent, and on comparing the figures for sodium hydroxide and sodium carbonate extracts it is seen that the sodium hydroxide extract is nearly one and a half times the sodium carbonate extract. The weight of the extracts increases as the stickiness increases.

Of organic solvents only alcohol was found to extract a certain fraction of manure and the humus from it. Alcoholic extracts are practically of the same order in each case. There is a tendency for the extract to increase with the degree of decomposition. The sugar content or the extract from ammonium carbonate on an average is greater than that from ammonium sulphate or sodium nitrate.

The significance of extraction with hydrogen peroxide is discussed in detail elsewhere [Shrikhande, 1933]. Much importance cannot be attached to the relationship between peroxide extract and stickiness. The nature of this extraction differs from others in the sense that this solvent oxidises some of the decomposed and synthesised material. All extracts if plotted graphically against stickiness give a straight line. In the rots with caseinogen there is an exception to the general order as mentioned above, all the extracts being disproportionately greater. This may be due to the fact that less caseinogen is decomposed by the organisms, and the remainder is dissolved by the solvents and returned unchanged in precipitates.

The initial p_H was highest with ammonium carbonate and lowest with ammonium sulphate. Sodium nitrate which was practically neutral at the start gave a final p_H of 10·0. The highest stickiness and decomposition were obtained with sodium nitrate and correspondingly lowest figures with ammonium sulphate, which maintained an acid reaction throughout. When, however, an equivalent amount of calcium carbonate was supplied to the ammonium sulphate rot, decomposition increased with an increase in stickiness. This leads to the conclusion that maximum stickiness is associated with alkaline conditions. This may mean either that the organisms responsible for stickiness are favoured by an alkaline medium, that there is a modification of the flora with the change in the environmental conditions or that the manifestation of the property of stickiness is enhanced by alkaline conditions. The p_H with all the three organic sources of nitrogen is practically of the same order (7·5) and similar figures for stickiness were obtained. The final high p_H with sodium nitrate is no doubt due to the utilisation of nitrate-N leaving excess of base. The slight lowering of p_H with ammonium carbonate may be ascribed to the use of nitrogen from ammonia and the liberation of CO_2.

Table IV. *Different extracts with physical test for stickiness and final* p_H *values of straws rotted by mixed floras with available nitrogen as urea and* $NaNO_3$ *at different temperatures.*

Expressed on 100 g. dry manure.

Temp. °C.	Source of N	Loss of D.M.	Physical test (g.)	Final p_H	Water extract	Na_2CO_3 extract	NaOH extract
15	Urea	7·2	414	7·8	17·1	7·7	13·2
	$NaNO_3$	12·3	2610	9·0	23·2	5·2	10·5
25	Urea	40·6	5020	8·0	29·6	11·9	16·0
	$NaNO_3$	30·2	3678	9·5	31·8	8·5	17·5
35	Urea	33·0	2278	7·5	25·3	11·6	19·7
	$NaNO_3$	52·5	7164	10·0	44·3	15·5	19·3
45	Urea	58·2	3650	8·5	38·7	18·1	19·8
	$NaNO_3$	40·9	7875	10·0	47·5	18·4	22·3

Series (c) and (d). Variation in temperature with urea and sodium nitrate as sources of nitrogen. Table IV contains results of extraction, final p_H and stickiness on samples of straws obtained as indicated above.

The losses of dry matter seem to increase with the increase in temperature. At 15° the decomposition is very poor in both cases, though sodium nitrate gives double that of urea. The decomposition obtained with urea at 25° is more than that at 35°. At 45° urea gives a decomposition of 58 %, which is much more than with sodium nitrate. These variations clearly indicate the different nature of the flora working at the different temperatures. The final p_H for urea varies between 7·8 at 15° and 8·5 at 45°. Similarly the p_H with sodium nitrate has increased from 9·0 at 15° to 10·0 at 45°. The initial p_H values were 6·45 and 6·35 respectively.

Stickiness. Even with a very small decomposition sodium nitrate produces quite an appreciable amount of stickiness, which increases with the rise in temperature. The maximum stickiness with urea as a source of nitrogen is produced at 25°.

Series (e). Degree of decomposition at 35° with sodium nitrate as the source of nitrogen. Table V indicates the relationship observed between the stickiness of the manure and the degree of decomposition when sodium nitrate was supplied

Table V. *Effect on the production of stickiness of modification of the* p_H *and the degree of decomposition.*

			Physical test in g.					
						p_H adjusted		
Source of N	Time in days	Loss of D.M. %	On manure	On original straw	Final p_H	To	With	On manure
$NaNO_3$	8	4·7	2050	1954	8·0	9·5	Na_2CO_3	3868
,,	16	23·1	4020	3903	9·0	9·5	Na_2CO_3	5521
						9·5	K_2CO_3	5123
						8·0	H_2SO_4	3648
						5·5	H_2SO_4	2305
,,	24	32·2	7834	5310	9·5	—	—	—
,,	32	34·8	8161	5320	9·5	8·0	H_2SO_4	5991
						7·0	H_2SO_4	4538
						5·5	H_2SO_4	3410

as the source of nitrogen. The physical test increases markedly as decomposition proceeds. There was, however, a possibility that this might be an effect of the changing reaction of the rot, which becomes progressively more alkaline and reaches finally a p_H of 9·5. To investigate this point the samples after 8 and 16 days' decomposition were adjusted to the final p_H of 9·5 by addition of sodium carbonate. This had the effect of increasing the figures for the physical test very appreciably, but not so much that they approached the level obtained at this p_H with longer periods of decomposition. There is therefore a direct relationship between the degree of decomposition of a manure and its stickiness, even when all the changes in the reaction have been taken into account. This point is more clearly brought out when the physical test is recalculated on a basis of original straw. The stickiness increased till the 24th day, after which further loss of organic matter was not accompanied by further production of apparent sticky material.

Further to demonstrate the effect of reaction, samples rotted for 32 days, having achieved the very high degree of stickiness indicated by a physical test of 8161 g., were acidified and the p_H reduced to 8·0, 7·0 and 5·5. This had the effect

of lowering the stickiness to 5970 g., 4538 g. and 3410 g. respectively. Similar adjustments of the p_H with sodium carbonate, potassium carbonate and sulphuric acid were made with the sample rotted for 16 days. These experiments indicate that the stickiness varies directly with the p_H within the limits tested. The relation between stickiness and reaction is represented graphically in Fig. 3.

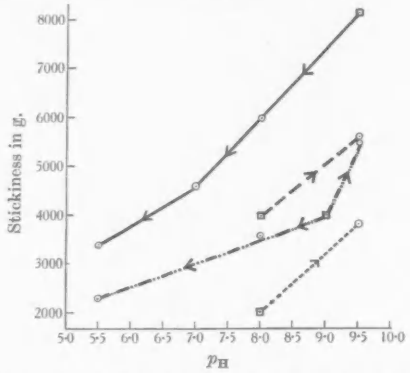

Fig. 3. Relationship between stickiness and reaction.

⊡ Initial point. → Changes due to alteration in p_H.
——— $NaNO_3$ 30 days. — — Urea 30 days.
—·—·— $NaNO_3$ 16 days. - - - - $NaNO_3$ 8 days.

Series (f). Adjustment of p_H *at 10·0 independently of the source of nitrogen.* Table VI deals with the effect on stickiness of the initial reaction of the material undergoing decomposition. Various sources of nitrogen were supplied and the

Table VI. *Effect of the initial* p_H *on the production of stickiness.*

Source of N	Initial p_H brought to 10·0 with	Final p_H	Loss of D.M. %	Physical test g.
Peptone	$MgCO_3$	8·0	35·3	3938
Peptone	Na_2CO_3	8·5	49·0	6490
Mould tissue as 1 % N	$MgCO_3$	8·0	37·0	4789
*Mould tissue as 1 % N	Na_2CO_3	8·0	52·2	4114
Mould tissue as 2 % N	$MgCO_3$	8·0	50·3	4863
Mould tissue as 2 % N	Na_2CO_3	8·5	56·5	5349
Urea	$CaCO_3$† (p_H:9·0)	8·0	39·0	3991
Urea	Na_2CO_3	8·5	42·3	6001

* Water-logged. † Final p_H brought to 10·0 by adding Na_2CO_3. Stickiness then equals 5620 g.

initial p_H of the straw adjusted to 10·0 with magnesium carbonate in one series and sodium carbonate in another. This particular reaction was chosen because the highest figure hitherto obtained for stickiness was found in a rot supplied with sodium nitrate which had attained finally that high degree of alkalinity. During fermentation the p_H values of both the series fell somewhat, those with magnesium carbonate more than those with sodium carbonate. The losses of dry matter and stickiness obtained were however invariably greater in the latter series. It is clear that if rots are adjusted initially to a high degree of

alkalinity the manure resulting is stickier than that which is usually obtained. Furthermore it appears that, given the correct p_H, sodium or potassium ions are more favourable than are calcium and magnesium.

The effect of sticky manure on soils.

Three soils of different composition were selected, a Rothamsted soil which owing to a high clay content is very heavy, a Woburn and a Cheshire soil, both of which are light, but the former more sandy. Two grams of the soil sample were mixed as uniformly as possible with one gram of the sticky manure and made into a paste with water. The dry matter of the mixed sample was adjusted to about 50 %. The physical test was carried out on one gram of this mixture as in the case of manures. Stickiness of the soils when wet and after drying was also determined to compare with the figures after mixing them with the sticky manure. The following table contains these figures.

	Physical test in g.		
Soil	Wet	Dried	When mixed with manure and dried
Rothamsted	431	924	947
Cheshire	0	424	829
Woburn	0	214	686

There is therefore a definite increase in the stickiness of light soils on mixing with such a manure.

The nature of the mucus.

Attempts to extract the sticky constituents of the manure with different solvents give unsatisfactory results. The usual method of precipitating gums from water and mildly alkaline extracts with absolute alcohol and Fehling's solution was also tried without success.

A sodium nitrate rot with mixed flora, which was definitely sticky to the touch and markedly so by the physical test, was extracted with cold water. The extract was colloidal. It was filtered twice through glass wool and finally through a filter-paper under suction. The extract was then precipitated with a few drops of HCl. The precipitate was coagulated by gentle heating on a hot plate and then filtered on a Büchner funnel. A very small fraction of this mixed with 1 g. of dry oat straw proved to be quite sticky. On drying the precipitate became very hard and gritty and lost its binding properties in part. Having thus established that the above precipitate contains a sticky constituent both when wet and dry, the following analytical figures are obtained on the extract.

Water extract	9·86 % on dry matter
Physical test for stickiness with 1 g. of the wet manure	4880 g.
Physical test with the wet extract ...	2555 g.
Physical test with the extract dried and then re-moistened	1575 g.
On hydrolysis with 3 % H_2SO_4 for 5 hours	
Apparent anhydroglucose	52·04 %
Anhydropectose	2·46 %
Protein	21·50 %
Ash	3·20 %
Total	79·20 %

After acid hydrolysis the extract reduced Fehling's solution and gave Selivanoff's test for fructose. On oxidation of a portion with concentrated nitric acid a precipitate was obtained consisting of colourless plates and micro-sandy crystals. The crystals were separated by shaking up with hot alcohol in which the plates dissolved. The insoluble residue had M.P. over 200°, and when distilled with conc. HCl yielded furfuraldehyde indicating the presence of mucic acid.

The extract appears therefore to be a mixture of carbohydrates and proteins, and no doubt in part consists of material extracted from the elaborated microbial tissue. The carbohydrate portion of the extract seems to consist largely of galactan, though indications have been obtained also of the presence of uronic acids and a little pentose.

Summary.

1. The conditions under which stickiness is produced in decomposing plant materials and manures have been investigated and some information obtained as to the nature of the substances contributing this property.

2. A physical test for evaluating the property of stickiness in manures has been described.

3. In the presence of a mixed natural flora, the chief factors involved in causing stickiness in decomposing straw are the source of nitrogen supplied, the initial and final reactions of the material and the degree of decomposition.

4. High values for stickiness are given with either sodium nitrate or mould tissues as the sources of nitrogen. This suggests that an alkaline reaction and an abundance of microbial tissue are essential in the production of stickiness during decomposition by mixed flora.

5. The final reaction of the manure profoundly influences the degree of stickiness, if at all appreciable. A p_H of 9·5 to 10·0, whether obtained by fermentation or by subsequent adjustment, seems to give the maximum stickiness. Sodium or potassium ions produce more stickiness than calcium or magnesium.

The author is indebted to Sir John Russell, Director of the Rothamsted Experimental Station, for placing at his disposal the facilities of the Station, and to Mr E. H. Richards, Head of the Fermentation Department, for suggesting the problem and for invaluable advice and criticism. Thanks are due to Dr A. G. Norman for his assistance and suggestions, and to Mr Scott Blair, of the Physics Department, for criticising the physical test.

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CCXIV. THE PRODUCTION OF MUCUS DURING THE DECOMPOSITION OF PLANT MATERIALS[1].

II. THE EFFECT OF CHANGES IN THE FLORA.

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The production of stickiness during the process of decomposition of cellulosic materials has been attributed by Hutchinson and Clayton [1919] to the activity of soil organisms such as *Spirochaeta cytophaga*. Since the time of Mitscherlich [1850] bacteria were considered to be the chief agents in the natural decomposition of plant materials until Konig [1904] suggested the greater importance of fungi. McBeth and Scales [1913] and Scales [1915] found fungi to be capable of using cellulose as the sole source of energy. It has been shown by Waksman [1926] and later confirmed by Norman [1929] that in the presence of sufficient available nitrogen, cellulose is the chief constituent to be decomposed. Waksman [1924] and Rege [1927] have shown that fungi are mainly responsible for the decomposition especially during the early stages.

Norman [1931] has studied the nitrogen transformations and changes in the carbohydrate constituents with pure cultures of fungi but no such study has so far been made of the subsequent action of bacteria after fungal attack, or of the simultaneous action of a bacterium with that of a fungus. Apart from the study of stickiness produced during decomposition, it was thought worth while to follow the ammonification and nitrogen changes and the losses in carbohydrate constituents at different stages of decomposition.

The work described in this paper was carried out in order to test whether mucus is produced during decomposition of straw with pure cultures of organisms. Straw was therefore decomposed with selected fungi, fungi and bacteria and bacteria alone. Analyses of the different constituents were made in order to find any correlation between the rates of their decomposition and the formation of mucus. The amount of mucus produced was measured by the physical test described in Part I [1933].

Scheme of work.

(1) Oat straw was rotted with pure cultures of fungi at 35° with ammonium carbonate as source of nitrogen and analysed at intervals of 8, 16, 24 and 48 days.

(2) Straw was rotted with pure cultures of bacteria: (i) *Mycobacterium agreste*, (ii) *Spirochaeta cytophaga*, and analysed at intervals of 8, 16, 24 and 48 days to see if any bacterium which, like the latter, produces a gummy colony on an artificial medium would produce stickiness in straw.

(3) Straw was rotted first for 48 days with fungi alone: (*a*) and then inoculated with *M. agreste* and analysed on the 8th, 16th, 24th and 48th days after inoculation; (*b*) as (*a*) but with the substitution of *S. cytophaga* for *M. agreste*.

[1] This paper is an abridged form of part of a thesis approved for the Degree of Doctor of Philosophy in the University of London.

(4) Straw was rotted with a fungus progressively and then inoculated with *S. cytophaga* on the 8th, 16th, 24th and 48th days, and analysed on the 8th and 40th days after inoculation with the latter.

(5) Straw was rotted simultaneously with fungus and *S. cytophaga* and analysed on the 8th, 16th, 24th and 48th days.

Methods.

Weighed quantities of straw were bottled and sterilised in an autoclave under 115 lbs. pressure for 45 minutes on two consecutive days. The treatment is undoubtedly drastic yet it is necessary for complete steriiisation. Available nitrogen was supplied as sterile ammonium carbonate in the proportion of 1 g. per 100 g. straw and the moisture was adjusted at about 80 %. A heavy inoculum of a suspension of the spores of the required organism in sterile water was then added to each bottle. Platings were made at the end of the experiments to test the purity of the organism. Fungal contamination was easily noticeable in most cases, since the straw was characteristically coloured by the spores of the fungi.

The following fungi were tested: *Trichoderma lignorum, Acremoniella olivaespora, Aspergillus niger, A. terreus, A. nidulans.*

The fungi tested were obtained from the stock cultures of the Mycology Department (isolated from the Rothamsted soils). They were maintained on agar slants in Waksman's medium. Before inoculation they were always grown afresh and in all the experiments described below cultures one week old were used.

Estimations of ammonia-N, total N, extraction with 90 % alcohol and treatment with hydrogen peroxide were carried out as in Part I.

(1) *Pentose units* (total furfuraldehyde). The standard method of Krober and Tollens was employed. The phloroglucide precipitate was not extracted with alcohol.

(2) *Cellulose* was estimated by Jenkins's method [1930].

(3) *Furfuraldehyde in cellulose* was determined as in (1).

The loss of dry matter (D.M.) as a result of decomposition is usually not a true index of the amount of decomposition. It does not consider the synthesis of new complexes by micro-organisms, which would modify to a considerable extent the actual amount of material decomposed. The cellulose and furfuraldehyde determinations would indicate how much of the loss in organic matter is represented by the loss in cellulose and hemicelluloses.

In any mature plant material, the cellulose, the polysaccharide associated with cellulose, or as it is termed by Hawley and Norman [1932], the "cellulosan," and the hemicelluloses are the three chief groups of biologically available material. The cellulose as isolated either by the Cross and Bevan chlorination method or by the Jenkins hypochlorite method is always associated with cellulosan. This cellulosan generally consists of pentose units. In the case of oat straw it is xylan as proved by Norman [1929]. This xylan can be determined by distilling the cellulose with 12 % HCl. A figure for "true" cellulose can then be obtained by deducting the figure for xylan. It is found that the decompositions of cellulose and the xylan associated with it run parallel. A figure for pentose units in the hemicelluloses can be obtained by subtracting from the total yield of furfuraldehyde that from the xylan associated with cellulose.

For the sake of comparison the figures recorded below are calculated on a basis of 100 g. of dry straw and interpreted in terms of cellulose, xylan associated with cellulose and pentose in hemicelluloses.

Experimental.

The same sample of oat straw was used throughout these experiments with pure cultures of organisms.

	%
Ash	9·04
Total N	0·35
Total furfuraldehyde	15·6
"True" cellulose	41·0
Furfuraldehyde on Jenkins's cellulose product	7·0
Xylan associated with cellulose	10·8
Furfuraldehyde due to pentose in polyuronides	8·6
Alcohol extract	3·46

Series I (Tables I and II).

Stickiness. No stickiness was observed with any fungi used for decomposition.

Carbohydrate constituents. Table I contains analyses of these constituents. *Acremoniella sp.* appears to be the most active fungus as judged from the loss of dry matter at the end of 48 days. *T. lignorum* seems to be the least active. The losses obtained follow practically the same order as those recorded by Norman [1931]. In general the hemicelluloses suffer a loss of about 60 % during 48 days. The hemicelluloses appear to decompose rapidly in the first few days and then remain at much the same level while the cellulose is being decomposed. On the whole it is obvious that the major part of the lost carbonaceous material is accounted for by the cellulose which is a more abundant food and energy source for the micro-organisms than the hemicelluloses.

Table I. *Decomposition of straws by various fungi at 35° at different intervals.*

Expressed on 100 g. original straw.

Fungus	Time in days	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
Acre. olivaespora	8	10·9	6·37	36·90	4·1	2·23
	16	21·2	5·19	27·81	13·1	3·41
	24	28·1	4·56	23·65	17·3	4·04
	48	41·2	3·32	16·17	24·8	5·28
A. nidulans	8	9·2	7·04	38·85	2·1	1·56
	16	15·6	6·01	35·64	5·3	2·59
	24	31·5	3·87	22·42	18·5	4·73
	48	38·9	3·23	17·02	23·9	5·37
A. terreus	8	11·0	6·98	37·66	3·3	1·62
	16	15·9	5·22	31·23	9·7	3·38
	24	31·9	4·66	24·09	16·9	3·94
	48	34·6	4·01	19·88	21·1	4·59
A. niger	8	11·2	4·98	37·37	2·6	3·62
	16	15·8	5·38	34·27	7·1	3·22
	24	23·1	4·86	26·25	14·7	3·74
	48	34·1	3·67	23·13	17·8	4·93
T. lignorum	8	9·3	4·23	38·29	2·7	4·37
	16	16·0	5·08	35·08	5·9	2·80
	24	24·4	5·02	27·82	13·1	3·58
	48	29·0	4·17	23·36	16·6	4·43

Table II. *Nitrogen content of straws rotted by pure cultures of fungi at 35° at different intervals.*

Expressed on 100 g. original straw.

Fungus	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
Acre. olivaespora	8	10·9	1·37	0·98	0·63	5·7
	16	21·2	1·32	1·29	0·93	4·4
	24	28·1	1·24	1·21	0·85	3·0
	48	41·2	1·19	1·17	0·84	2·0
A. nidulans	8	9·2	1·14	0·62	0·27	2·9
	16	15·6	0·92	0·72	0·37	2·3
	24	31·5	0·94	0·92	0·56	1·8
	48	38·9	1·12	1·09	0·73	1·8
A. terreus	8	11·0	1·22	0·75	0·40	3·7
	16	15·9	1·12	0·99	0·64	4·0
	24	31·9	0·85	0·82	0·51	1·6
	48	34·6	1·02	1·01	0·66	1·9
A. niger	8	11·2	1·20	0·82	0·46	4·1
	16	15·8	1·11	1·03	0·65	4·1
	24	23·1	1·24	1·22	0·86	3·7
	48	34·1	1·02	1·02	0·86	2·5
T. lignorum	8	9·3	1·23	0·86	0·50	5·4
	16	16·0	1·09	0·93	0·65	4·8
	24	24·4	1·25	1·24	0·88	3·6
	48	29·0	1·25	1·24	1·01	3·4

Nitrogen immobilisation. From Table II it can be seen that more than 50 % of the inorganic nitrogen has been used up by the organisms except *A. nidulans* in the first 8 days. The power of an organism in building up the microbial protein can best be judged from the nitrogen factor [Richards and Norman, 1931]. *T. lignorum* seems to be the best from the point of view of nitrogen immobilisation and *A. nidulans* the poorest. Norman [1931] also recorded a low nitrogen factor for *A. nidulans* compared with *A. niger* and *A. terreus*. As compared by their nitrogen equivalents [Norman, 1931] *A. nidulans* seems to be the most efficient and *Trichoderma* the least. *A. niger* differs markedly from *A. nidulans* and *A. terreus* though belonging to the same genus.

Series II (Tables III, IV and V).

Stickiness. Both the organisms produce a negligible amount of stickiness. The figures for *S. cytophaga* are contrary to expectation. Since it is an organism which produces a gummy colony on agar media, it was supposed that it might also give a rotted product showing stickiness.

Table III. *Physical test, alcoholic extracts and the H_2O_2 treatment of straws rotted with pure cultures of bacteria at 35° at different intervals.*

Expressed on 100 g. dry manure.

Bacterium	Time in days	Loss of D.M.*	Alcohol extract	H_2O_2 extract	Loss of O.M. after H_2O_2 treatment	Physical test (g.)
S. cytophaga	8	7·5	8·40	7·0	10·1	108·6
	16	12·2	6·87	10·1	11·4	108·2
	24	13·0	7·48	13·9	11·4	212·3
	48			Infected		
M. agreste	8	4·8	7·05	13·7	12·6	101·3
	16	8·5	7·41	12·1	11·4	0·0
	24	14·2	7·51	9·7	13·8	334·1
	48			Infected		

* On 100 g. original straw.

Extractions with alcohol and hydrogen peroxide (Table III). On an average the alcoholic extract is the same for both of the organisms with no further change in the degree of decomposition. There is an increase in the hydrogen peroxide extract with *S. cytophaga* whereas the order is reversed with *M. agreste*.

Carbohydrate constituents (Table IV). The decomposition is practically of the same order with both the organisms at the end of 24 days. The bottles for the 48th day were infected and hence rejected. The cellulose lost with *Mycobacterium* is nearly one and a half times that destroyed by *Spirochaeta*. This was

Table IV. *Decomposition of straw by bacteria at 35° at different intervals.*

Expressed on 100 g. original straw.

Bacterium	Time in days	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
S. cytophaga	8	7·5	5·60	38·82	2·1	3·00
	16	12·2	6·11	36·76	4·2	2·59
	24	13·0	5·31	36·29	4·7	3·29
	48			Infected		
M. agreste	8	4·8	6·06	39·14	1·8	2·54
	16	8·5	6·02	36·43	4·5	2·58
	24	14·2	5·95	34·31	6·6	2·65
	48			Infected		

entirely unexpected, since the former is not ordinarily regarded as being a cellulose-decomposing organism and does not develop on a cellulose-agar plate, but, as pointed out by Norman [1930], the ability to utilise cellulose in the presence of other available carbohydrates is more common than is generally supposed. On the other hand *Spirochaeta* destroys hemicelluloses to a considerably greater extent than does *Mycobacterium*. Both organisms compare poorly with fungi in destructive action as regards organic matter.

Nitrogen immobilisation. The nitrogen figures are recorded in Table V. *Mycobacterium* builds up more microbial tissue than does *Spirochaeta* as seen

Table V. *Nitrogen content of straw rotted with pure cultures of bacteria at 35° at different intervals.*

Expressed on 100 g. original straw.

Bacterium	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
S. cytophaga	8	7·5	1·268	0·75	0·39	5·2
	16	12·2	1·000	0·64	0·29	2·3
	24	13·0	0·957	0·80	0·46	3·5
				Infected		
M. agreste	8	4·8	1·290	0·93	0·58	11·8
	16	8·5	1·040	0·66	0·30	3·5
	24	14·2	1·180	0·97	0·60	4·2
				Infected		

from the nitrogen factor. Compared with fungi, bacteria appear to be much slower in building up microbial protein. *Spirochaeta* consumes more carbohydrate per g. of nitrogen than *Mycobacterium* as shown by the nitrogen equivalent.

Table VI. *Decomposition of straw rotted first with pure cultures of fungi for 48 days and then inoculated with* M. agreste *and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
T. lignorum	8	30·1	3·52	24·74	16·2	5·08
	16	44·5	2·34	12·92	28·0	6·26
	24	49·1	2·46	11·86	29·1	6·14
	48	39·0*	3·61	21·14	19·8	5·00
A. niger	8	35·7	3·74	20·20	20·8	4·86
	16	42·0	3·04	15·99	25·0	4·56
	24	36·0	3·63	17·52	23·4	4·97
	48	41·0	3·06	17·70	23·3	4·74
A. nidulans	8	25·0	4·39	29·53	11·4	4·21
	16	28·0	4·26	27·38	12·6	4·34
	24	36·0	2·94	18·98	22·0	5·66
	48	38·7	3·79	20·96	20·0	4·81
Acre. olivaespora	8	32·4	4·06	25·18	15·8	4·54
	16	43·3	3·14	16·48	24·5	5·46
	24	43·4	2·94	15·59	25·4	5·66
	48	44·5	3·60	18·32	22·6	5·00
A. terreus	8	31·2	3·57	23·12	17·8	5·03
	16	36·9	3·55	19·00	22·0	5·03
	24	37·5	3·91	21·19	19·8	4·69
	48	39·7	3·42	19·81	21·2	5·18

* Short of moisture.

Table VII. *Nitrogen content of straw rotted first with pure cultures of fungi for 48 days and then inoculated with* M. agreste *and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Total N	Organic N	N factor	N equiv.
T. lignorum	8	30·1	1·01	1·00	0·58	3·3
	16	44·5	1·22	1·21	0·70	1·5
	24	49·1	1·14	1·10	0·77	1·5
	48	39·0*	0·91	0·88	0·55	1·4
A. niger	8	35·7	1·08	1·06	0·72	2·0
	16	42·0	1·25	1·19	0·84	2·0
	24	38·0	0·95	0·94	0·67	1·7
	48	41·0	0·92	0·90	0·54	1·3
A. nidulans	8	25·0	0·97	0·95	0·62	2·5
	16	28·4	0·97	0·96	0·60	2·1
	24	36·9	1·16	1·14	0·80	2·1
	48	38·7	0·97	0·95	0·60	1·5
Acre. olivaespora	8	32·4	1·00	0·98	0·70	2·1
	16	43·3	1·25	1·19	0·84	1·9
	24	43·4	1·19	1·13	0·82	1·9
	48	44·5	1·10	1·08	0·73	1·6
A. terreus	8	31·2	1·06	1·05	0·69	2·2
	16	36·9	0·97	0·96	0·59	1·6
	24	37·5	0·87	0·86	0·51	1·3
	48	39·7	0·95	0·88	0·53	1·3

* Short of moisture.

Series III (*a*) (Tables VI and VII).

Stickiness. The rots showed no stickiness, indicating that *M. agreste* is unable to synthesise sticky material even after acting upon the elaborated fungal tissue.

Carbohydrate constituents (Table VI). *M. agreste* following upon *Trichoderma* gives the best decomposition and a loss of 49·1 % organic matter in only 24 days. The losses with the *Aspergilli* are practically of the same order. The rate of decomposition in the first eight days after inoculation with all the fungi is greater than in any subsequent periods. The average loss of cellulose with *Mycobacterium* following upon the five different fungi ranges from 20 to 23 %. Further decomposition does not affect the pentose unassociated with cellulose showing that all the available pentoses have already been removed by the fungi.

Nitrogen immobilisation (Table VII). With *Mycobacterium* following on each of the fungi, there is a drop in the nitrogen factor after the 16th day in general, which is due to the ammonification or as it is termed by Jensen [1929] the "mineralisation" of a portion of the fungal protein.

Series III (*b*) (Tables VIII, IX and X).

Stickiness. The results for stickiness are given in Table VIII and are highly significant. No stickiness was found with fungi on the 48th day, but by the subsequent action of *S. cytophaga* for 8 days only, the pull required to separate

Table VIII. *Physical test, alcohol extracts and the H_2O_2 treatment of straws rotted first with pure cultures of fungi and then inoculated with* S. cytophaga *and analysed at different intervals.*

Expressed on 100 g. dry manure.

Fungus	Days after inoculation	Loss of D.M.	Alcohol extract	H_2O_2 extract	Loss of O.M. with H_2O_2	Physical test (g.)
T. lignorum	8	39·2	10·50	13·1	22·0	3495
	16	39·7	8·50	12·1	22·3	6435
	24	42·6	8·87	12·8	23·0	3958
	48	57·7	12·20	29·0	26·0	6550
A. niger	8	41·4	9·90	13·3	24·7	6305
	16	45·0	8·90	17·5	25·6	4242
	24	46·0	10·20	26·6	24·7	7134
	48	48·3	9·20	24·7	25·0	6145
A. nidulans	8	35·8	8·80	11·7	22·8	5197
	16	41·2	8·60	16·8	26·2	6796
	24	43·4	9·04	13·8	30·0	5869
	48	43·5	9·05	22·8	22·0	5290
Acre. olivaespora	8	43·1	9·14	13·3	24·0	4425
	16	43·2	8·02	13·8	23·2	7866
	24	45·0	8·56	21·0	25·0	4995
	48	46·3	7·64	15·0	25·0	6904
A. terreus	8	37·5	7·64	14·2	23·7	6439
	16	41·9	7·90	11·8	26·4	5970
	24	45·4	7·64	15·0	25·0	8386
	48	51·3	7·82	26·4	25·0	6442

The final p_H of all the samples was between 7·0 and 7·5.

the two plates was at once raised by from 3 to 6 kg. varying with the nature of the fungus. This obviously means that *Spirochaeta* has synthesised some organic matter which is responsible for the stickiness. Since it was observed in Series II that *Spirochaeta* alone could hardly produce any stickiness, one is naturally led

to believe that the sticky material has some relation to the previous action of the fungus. Again, *Mycobacterium* did not produce any stickiness even after a previous fungal decomposition, suggesting that the production of stickiness has also some relation to the nature of the bacterium taking part in the decomposition.

Extractions by alcohol and hydrogen peroxide (Table VIII). The averages for the alcohol extracts differ in each case, the maximum being 10 % with *Spirochaeta* following on *Trichoderma* and 7·7 % on *A. terreus*. These figures are interesting when compared with stickiness. The maximum stickiness obtained is with the latter combination, whereas the former gives the least. On the whole the alcoholic extracts are greater than those with *Mycobacterium*. The higher solubility of the sticky material in alcohol indicates its possible non-gummy and non-dextrin nature. The losses of organic matter on treatment with peroxide are in general proportional to the losses in dry matter.

Carbohydrate constituents (Table IX). Greatest decomposition is obtained by *Spirochaeta* following on *Trichoderma* and *A. terreus* and amounts to 51·7 and 51·3 % respectively. In these two cases there is a big jump in the loss of dry

Table IX. *Decomposition of straws rotted first with pure cultures of fungi for 48 days and then inoculated with* S. cytophaga *and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of furfuraldehyde from non-cellulosic constituents
T. lignorum	8	39·2	3·16	14·61	5·24
	16	39·7	3·06	18·26	5·54
	24	42·6	3·17	14·89	5·43
	48	51·7	2·21	10·58	6·39
A. niger	8	41·4	2·88	14·57	5·72
	16	45·0	2·44	13·78	6·16
	24	46·0	2·56	13·52	6·04
	48	48·3	2·39	12·03	6·21
A. nidulans	8	35·8	3·88	18·46	4·72
	16	41·2	2·89	13·65	5·71
	24	43·4	3·09	16·28	5·51
	48	43·5	2·94	13·89	5·66
Acre. olivaespora	8	43·1	2·86	13·79	5·74
	16	43·2	3·41	15·85	5·19
	24	45·0	2·66	13·42	5·94
	48	46·3	2·20	13·06	6·40
A. terreus	8	37·5	3·15	16·45	5·45
	16	41·9	2·86	14·61	5·74
	24	45·4	2·20	13·74	6·40
	48	51·3	2·06	12·58	6·54

matter between the 24th and 48th days after the inoculation with *Spirochaeta*, but with the remainder the decomposition appears to be steady. The average loss of cellulose is about 26 % in each case. The average loss of pentose not associated with cellulose is also practically the same. These losses are not significantly higher than those given by fungi alone at the end of 48 days, indicating that no available hemicelluloses are left after fungus action.

Nitrogen immobilisation (Table X). Here again there is a drop in the nitrogen factor as the decomposition proceeds. This fall is more marked with *Spirochaeta* than with *Mycobacterium*. The former appears more efficient than the latter per unit of nitrogen as judged by the nitrogen equivalent.

Table X. *Nitrogen content of straws rotted first with fungi for 48 days and then inoculated with* S. cytophaga *and analysed at different intervals.*

Expressed on 100 g. original straw.

Fungus	Days after inoculation	Loss of D.M.	Total N	Organic N	N factor	N equiv.
T. lignorum	8	39·2	1·26	1·24	0·89	2·2
	16	39·7	1·13	1·11	0·77	1·8
	24	42·6	1·10	1·08	0·73	1·7
	48	51·7	1·12	1·09	0·74	1·4
A. niger	8	41·4	1·35	1·30	0·96	2·3
	16	45·0	1·24	1·18	0·82	1·8
	24	46·0	1·26	1·18	0·83	1·8
	48	48·3	1·20	1·11	0·76	1·5
A. nidulans	8	35·8	1·22	1·20	0·91	2·5
	16	41·2	1·39	1·31	0·84	2·0
	24	43·4	1·14	1·12	0·77	1·7
	48	43·5	1·16	1·13	0·80	1·8
Acre. olivaespora	8	43·1	1·31	1·23	0·95	2·1
	16	43·2	1·15	1·11	0·78	1·8
	24	45·0	1·13	1·10	0·76	1·7
	48	46·3	1·25	1·23	0·82	1·7
A. terreus	8	37·5	1·34	1·27	0·99	2·6
	16	41·9	1·28	1·25	0·85	2·0
	24	45·4	1·23	1·19	0·84	1·7
	48	51·3	1·20	1·17	0·81	1·6

Series IV (Tables XI and XII).

Stickiness (Table XI). Fungi alone produce no stickiness, but it develops as soon as *S. cytophaga* is inoculated even when the fungus had a start of only a week. This further emphasises the necessity of the presence of fungal tissue for

Table XI. *Decomposition of straw rotted first with fungus for different periods and then inoculated with* S. cytophaga *and analysed on the 8th and 40th days after inoculation.*

Expressed on 100 g. original straw.

Organism	Days of fungus action	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents	Physical test (g.)
T. lignorum	8	27·8	4·27	23·52	17·4	3·33	3771
+	16	36·5	2·93	16·60	24·4	5·67	4452
S. cytophaga	24	37·6	3·36	14·10	26·9	5·24	4735
for 8 days	48	48·5	2·18	9·30	31·7	6·42	4143
T. lignorum	8	46·2	2·74	10·24	30·7	5·86	5196
+	16	47·9	2·32	9·45	31·5	6·28	1998
S. cytophaga	24	48·9	1·76	10·03	30·9	6·94	1880
for 40 days	48	54·9	1·36	7·97	33·0	7·24	2077

the production of stickiness. The stickiness was practically constant at about 4275 g. when *Spirochaeta* acted for only 8 days whereas it varied considerably when it acted for 40 days.

Carbohydrate constituents (Table XI). By comparing the figures in Series I for losses in dry matter with those of *Trichoderma* alone in Table I an estimate can be obtained as to the further decomposition due to *Spirochaeta* in conjunction with the fungus. For instance, the fungus working alone decomposed 16 % of dry matter in 16 days, whereas if working in association with the bacterium for the second 8 days, *i.e.* a total of 16 days, a decomposition of 27 % was obtained.

Obviously this extra loss must be ascribed to the action of the bacterium. For 48 days with the fungus and 8 days with the bacterium the decomposition amounted to 48 %, which is very close to that obtained with the fungus for 16 days and the bacterium for 40 days. The loss of dry matter for 48 days with the fungus and 40 days with the bacterium is about 55 %, which compares well with 51 % obtained in Series III (*b*) for a total period of 96 days. The losses in carbohydrate constituents run parallel with the losses in dry matter.

Nitrogen immobilisation (Table XII). The increase in ammonification with the period of incubation explains the drop in the nitrogen factor. *Spirochaeta*

Table XII. *Nitrogen content of straws rotted first with fungus for different periods and then analysed on the 8th and 40th days after inoculation with* S. cytophaga.

Expressed on 100 g. original straw.

Organism	Days of fungus action	Loss of D.M.	Total N	Organic N	N factor	N equiv.
T. lignorum	8	27·8	1·49	1·45	1·09	3·9
+	16	36·5	1·36	1·21	0·86	2·3
S. cytophaga	24	37·6	1·32	1·18	0·82	2·2
for 8 days	48	48·5	1·29	1·23	0·89	1·8
T. lignorum	8	46·2	1·33	1·30	0·94	2·0
+	16	47·9	1·38	1·34	0·98	2·0
S. cytophaga	24	48·9	1·30	1·23	0·88	1·8
for 40 days	48	54·9	1·21	1·16	0·81	1·5

has accordingly decomposed the fungus protein for its nitrogen requirements; the excess of ammonia is volatilised, thus accounting for the nitrogen losses.

Series V (Tables XIII and XIV).

Stickiness. The figures for stickiness indicate that the joint action of the two organisms from the very start is unfavourable for its production. The average of 240 g. for stickiness is far below the figures recorded where the fungus had acted alone for at least a week. The low value for stickiness may be accounted for by the failure of the fungus to build up the microbial tissue which appears to be of prime importance in the production of stickiness.

Carbohydrate constituents (Table XIII). The rate of decomposition appears to be very steady, giving a maximum of about 28 % which is approximately that of *Trichoderma* alone for 48 days. In fact, greater decomposition was expected

Table XIII. *Decomposition of straw with fungus and* S. cytophaga *together for different intervals.*

Expressed on 100 g. original straw.

Organism	Time in days	Loss of D.M.	Furfuraldehyde from pentose groups not in cellulose	"True" cellulose	Loss of "true" cellulose	Loss of furfuraldehyde from non-cellulosic constituents	Physical test (g.)
T. lignorum	8	6·9	6·69	37·93	3·0	1·91	139
+	16	11·9	6·52	35·41	5·5	2·08	163
S. cytophaga	24	14·5	5·02	33·77	7·2	3·58	395
together	48	27·9	4·68	20·62	20·3	3·92	266

considering the cellulose-decomposing nature of both the organisms. This low decomposition may partly be accounted for by the apparent failure of the fungus to grow in presence of *Spirochaeta*. The cellulose loss is in proportion to the loss of dry matter. A loss of 13 % dry matter between the 24th and 48th days

corresponds with 13 % loss of cellulose during the same period. The greater part of the loss of organic matter is thus accounted for by the loss in cellulose. There is hardly any increase in the loss of pentose between 24 and 48 days, no doubt due to the inactivity of the fungus.

Table XIV. *Nitrogen content of straws rotted with fungus and* S. cytophaga *together for different intervals.*

Expressed on 100 g. original straw.

Organism	Time in days	Loss of D.M.	Total N	Organic N	N factor	N equiv.
T. lignorum	8	6·9	1·37	0·95	0·60	8·6
+	16	11·9	1·28	1·00	0·64	5·4
S. cytophaga	24	14·5	1·04	0·84	0·48	3·3
together	48	27·9	1·30	1·27	0·92	3·3

Nitrogen immobilisation (Table XIV). The organic nitrogen is lower than that obtained with the fungus alone. This may be explained by the inactivity of the fungus. The low nitrogen factor in the early stages may be due to the poor synthesis of fungal tissue in the presence of the bacterium.

Discussion and conclusions.

It appears from these decomposition studies that the production of stickiness in straws depends upon the presence of fungal tissue and the nature of the bacterium. Since *S. cytophaga* has a comparatively low optimum temperature for development, it appears that under the high temperature conditions of fermentation in a manure heap some other organisms must be the main causes of production of stickiness. Stickiness seems to have no correlation with the disappearance of any particular carbohydrate constituent during decomposition.

Although *S. cytophaga* produces gum on a synthetic medium, it fails to produce any stickiness while working upon straws, but in the presence of fungal tissue *S. cytophaga* produces stickiness. It is possible therefore that there is a fundamental difference between the sticky material synthesised by *S. cytophaga* while working upon straw previously decomposed by fungi and the gum it produces on an artificial medium which contains no fungal tissue. Obviously some sticky material other than the bacterial gum is synthesised during decomposition of straw by the interaction between the fungal tissue and the bacterium.

The simultaneous inoculation of fungus and *S. cytophaga* on sterile straw was not very successful either from the point of view of production of stickiness or of general decomposition. The fungus developed with difficulty and two further inoculations were necessary. This may be due either to competition for food material between the fungus and the bacterium or the retarding effect of the end-products of the bacterial action on the growth of the fungus. No counts were made to test this possibility, but it has been observed by Rege [1927] and others that fungi which bring about the initial decomposition of straw eventually disappear and are replaced by bacteria.

Summary.

(1) A number of common soil fungi and two cellulose-decomposing bacteria in pure culture and in different associations have been tested with reference to the production of stickiness and general decomposition.

(2) These fungi and bacteria, while working independently of each other, do not produce stickiness irrespective of the nature of the bacteria.

(3) Fungus decomposition followed by the action of *Mycobacterium agreste* does not produce stickiness.

(4) Fungus decomposition followed by the action of *Spirochaeta cytophaga* produces stickiness.

(5) Progressive decomposition with a fungus and subsequent inoculation with *S. cytophaga* at different stages produce stickiness even if the period of action of the fungus was brief.

(6) Simultaneous inoculations of fungus and *S. cytophaga* produce very little stickiness.

(7) The amount of decomposition effected, the losses in carbohydrate constituents and the nitrogen immobilisation in each case were determined. All the substances studied were removed approximately in proportion to the apparent losses of dry matter.

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CCXV. THE GLUTEN-DISSOLVING FERMENT OF WHEAT AND BARLEY SEEDS.

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In the winter of 1931–32 Miss Kozmin drew our attention to the fact that gluten washed out from wheat grains damaged by the bites of the "tortoise bug" (*Eurygaster* species) within 2 hours at the most following the washing out turns into a liquid. This phenomenon was described by Kozmin [1929; 1930] and Berliner and Koopman [1929], both of whom have suggested that it was probably a case of the action of strong proteolytic ferments. However, testing by the usual method, *i.e.* by autolysis of 5 g. of flour from the affected grain for 6 hours at p_H 4·7 and a temperature of 34° showed only a slight increase of the free amino-groups in comparison with the autolysis of flour from normal grain; the normal grain gave an increase of 2·1 mg. and the damaged grain 2·44 mg. Amino-nitrogen was determined by the indirect method of titration of the carboxyl groups by the Willstätter method, and a difference of 0·34 mg. on a 5 g. aliquot could not be considered decisive. After 48 hours of autolysis there was hardly a change in the difference between normal and damaged flour. The total nitrogen content of the 5 g. of flour being about 120–130 mg. an increase of amino-nitrogen of about 0·3–0·6 mg. certainly could not cause such a liquefaction of the gluten as was mentioned above.

Our next hypothesis was to assume the existence of a specific ferment in the damaged grain dissolving only the proteins of the gluten without splitting the amino-linkages in the polypeptide chains. In order to verify this assumption we have prepared a water extract from the damaged grain by placing in a thermostat a mixture of 1 g. of flour, 5 cc. of water and 1 cc. of toluene, allowing it to stand there for 48 hours at a temperature of 33–35° and filtering. From the flour of the undamaged grain fresh gluten was washed off. Aliquots of 1 g. were weighed into Erlenmeyer flasks and to each were added 20 cc. of acetate buffer p_H 4·7, 5 cc. of the ferment extract and 1 cc. of toluene. One part of the flasks was placed for 10 minutes over a water-bath and then in a thermostat for 24 hours. The contents of the second part were analysed immediately and the third part was placed in a thermostat without heating. In the thermostat were also placed flasks containing only gluten without the extract and others containing only the extract without gluten. For analyses the contents of the flasks were filtered through an ashless filter directly into a Kjeldahl flask and carefully washed out. The residue on the filter was digested according to the Kjeldahl method and so was the filtrate mixed with the water used for the washing out. The analyses gave the following results.

	Nitrogen in mg.			
	In filtrate	In residue	Total	
Without heating, 24 hours at 33–35°	20·13	50·16	70·29	
Heated, 24 hours at 33–35°	15·63	53·56	69·19	
Without heating, determined immediately	16·83	53·46	70·29	
Gluten alone after 24 hours	6·58	50·37	56·95	70·26
Extract alone after 24 hours	13·31	—	13·31	

After 24 hours' digestion about 8 % of the nitrogen of the gluten passed into the filtrate.

An experiment was undertaken under the same conditions on the effect upon the gluten of a diastatic preparation ("maltin" of Kahlbaum, *viz.* a preparation from sprouted barley). The increase of amino-nitrogen titratable by the Sørensen method amounted after 24 hours only to 1·94 mg. The variation in solubility is expressed by the following values.

	Nitrogen in mg.			
	In filtrate	In residue	Total	
Without heating, 24 hours at 33–35°	35·57	26·82	62·39	
Without heating, determined immediately	4·28	55·87	60·15	
Heated up to 100°, 24 hours at 33–35°	13·64	46·73	60·37	
Gluten alone, 24 hours at 33–35°	12·18	44·22	56·40	62·49
Diastase alone, 24 hours at 33–35°	3·26	2·83	6·09	

The results obtained after 6 hours' treatment were also sufficiently definite, *viz.*:

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 6 hours	16·57	43·50	60·07
Without heating, determined immediately	4·39	56·16	60·55
Heated up to 100°, 6 hours	4·53	55·31	59·84

A still more energetic action was observed in an alkaline medium at p_H 8·0. The results of a corresponding experiment carried on for 24 hours at a temperature of 33–35° with gluten and maltin were the following:

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours	47·52	13·66	61·18
Without heating, immediately	5·82	54·49	60·31
Heated to 100°, 24 hours	6·90	55·07	61·97
Gluten alone, 24 hours	4·31	53·70	58·01

The effect upon the gluten at p_H 8·0 of the extract from the grain damaged by the bug gave on the whole the same results as at p_H 4·7.

	Nitrogen in mg.		
	In filtrate	In residue	Total
Without heating, 24 hours	9·16	53·77	62·93
Without heating, immediately	6·47	55·93	62·40
Heated to 100°, 24 hours	6·90	56·22	63·12

The next step was the study of the effect of the dissolving ferment upon the components of the gluten: glutenin and gliadin. The preparations of these proteins were made according to Kiesel *et al.* [1931] from normal wheat flour, p_H 4·7 (acetate buffer). Dry preparations of protein (0·2 g.) were used. The increase of amino-nitrogen titratable by Sørensen's method amounted in the

glutenin for the 24 hours to 3·24 mg. The effect of diastase ("maltin") gave the following results:

	Glutenin Nitrogen in mg.					Gliadin Nitrogen in mg.				
	In filtrate		In residue		Total	In filtrate		In residue		Total
Without heating, 24 hours	19·83 18·48	19·17	15·03 15·55	15·29	34·86 34·03	31·21 31·27	31·24	5·11 5·55	5·33	36·22 36·82
Without heating, immediately	14·53 14·93	14·73	20·14 19·45	19·29	34·67 34·48	20·09 21·41	20·66	15·65 15·38	15·52	35·74 36·79
Heated to 100°, 24 hours	8·07 7·30	7·69	26·45 27·11	26·28	34·52 34·41	*18·44 *20·08	19·26	*15·24 *13·90	14·57	*33·68 *33·98
Protein alone, 24 hours	3·21 2·98	3·09	28·76 28·60	0·68	31·97 31·58	19·54 19·05	19·31	14·22 16·22	15·22	33·76 35·27
Diastase alone, immediately	1·51 1·47	1·49	1·22 1·22	1·22	2·73 2·69	1·51 1·47	1·49	1·22 1·22	—	2·78 2·69
Diastase alone, 24 hours	1·83 1·83	1·83	0·81 0·88	0·85	2·64 2·71	1·83 1·83	1·83	0·81 0·88	0·85	2·64 2·71

* Gliadin without the ferment immediately after the experiment was started.

The dissolving effect upon gliadin is considerably greater than upon glutenin.

The enzymic nature of the disintegration of the protein without an increase of the free amino-groups or at least with a considerably smaller accumulation of amino-groups in comparison with the amounts of nitrogen rendered soluble is sufficiently clear from the above experiments. It is hard to imagine what linkages are really being split in the protein molecule during this reaction. It may be believed, however, as is thought by Astbury and Wood [1931], that the protein molecule consists of parallel polypeptide chains connected with each other by means of additional valencies into compound aggregates. Such connections may be formed between 2, 3 or 6 polypeptide chains, which is quite in agreement with the fact found by Svedberg [1931] that the molecular weight of the protein is either equal to 34,500 or 2, 3 or 6 times this. The breaking of the bond between the different polypeptide chains may cause a change of the aggregate state of the protein without freeing new amino-groups. The appearance of the latter is connected with the presence in the ferment preparations of real proteases.

Summary.

1. The gluten of flour from grain damaged by the "tortoise bug" rapidly changes its aggregate condition without any noticeable increase of the free amino-groups.

2. The disintegration of the gluten and of the component parts of it, glutenin and gliadin, which is connected only with a change of the aggregate state of the protein, is a process of an enzymic nature.

3. The corresponding ferment, which is representative of an entirely new type of disaggregating ferments, acts at both acid and alkaline reactions and is found in the seeds of wheat damaged by the "tortoise bug" and in the diastatic preparation from malt.

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CCXVI. SOME LIMITATIONS OF THE CARIUS DIGESTION FOR THE MEASUREMENT OF CHLORIDE IN BIOLOGICAL MATERIAL.

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In the quantitative determination of chloride of desiccated biological tissues, particularly those containing fats, it has been amply demonstrated that complete recovery is not obtainable by the method of open Carius digestion with acid. On the other hand the use of preliminary alkaline digestion followed by the open Carius method has been shown by one of us [1933] to give quantitative recovery.

The failure to recover the chloride completely by the Carius method has been interpreted by some, particularly by Morris and Morris [1930; 1932] as due to an actual loss of chloride in some volatile form during the drying process. We have shown that this interpretation is incorrect since we have reported [1931] that if desiccated blood be allowed to stand in contact with water before the Carius digestion, almost complete recovery of chloride results; and more recently one of us has shown [1933] that subjecting dried tissues to preliminary alkaline digestion yields complete recovery as compared with established methods of analysis. The confusion between "diminution in chloride measurement" and an actual loss of chloride persists in the recent article by Norris and Ampt [1933] who have failed to recognise that our experiments indicated not a loss of chloride but rather a failure to measure it.

It was suggested by us that traces of halide may distil off during the digestion of blood and tissues with $AgNO_3$ and HNO_3, though in quantities insufficient to account for the diminution in chloride measurement which we reported in the analysis of desiccated biological materials. The following experiments were devised to measure the amount of halide lost during the Carius digestion, using blood as a test material.

Samples of wet and dried blood were digested with $AgNO_3$ and HNO_3 in a pyrex flask connected by ground glass joints to the digestion flask of a Thompson-Oakdale apparatus[1]. The fumes given off from the digestion mixture were passed through hot concentrated H_2SO_4 in the digestion flask of the Thompson-Oakdale apparatus, and after having passed through quartz tubing maintained electrically at cherry-red heat they were collected in a solution of Na_2HAsO_3. The residual fumes following the Carius digestion were drawn through the Thompson-Oakdale

[1] In some of the experiments a similarly modified Willard-Thompson apparatus was employed.

apparatus in a stream of chloride-free air. The Na_2HAsO_3 solution was neutralised with HNO_3, and the halide precipitated with $AgNO_3$. The results of the analyses are given in Table I. The estimations of the halide recovered from the fumes were made by comparing the turbidity of the unknown with that of freshly prepared suspensions of AgCl. By comparison of turbidity we were able to estimate any chloride that might be lost up to 3 *mM* per litre of blood within $\pm 0{\cdot}1\,mM$.

Table I.

Material	Remarks	Maximum halide recovered from fumes from Carius digestion. Milli-equiv./l. wet sample	Chloride analyses. Milli-equiv./l. wet sample
Ox blood 16	Wet samples dried in flasks at 110° for 24 hours	0·2 0·5	80·1 66·8
Ox blood 18	Wet samples dried and subjected to preliminary alkaline digestion	0·3	—
Human blood	(*a*) Wet samples	0·5	—
	(*b*) Dried and finely pulverised samples	1·0	—
	(*c*) Samples dried in flasks	1·8	—
NaCl sol. 100 milli-equiv./l.	—	0·1 (same as reagent blank)	—
Human blood	(*a*) Wet samples	—	83·2
	(*b*) Dried samples subjected to preliminary alkaline digestion	—	83·8
	(*c*) Wet samples digested in flasks connected with stoppered reflux condensers	—	83·0
	(*d*) Dried samples digested in flasks connected with stoppered reflux condensers	—	68·8

It will be seen from Table I that the minute amount of halide which we were able to recover from the fumes during the Carius digestion of blood accounts only to a very small extent for the failure to measure chloride completely by the direct Carius method with dried blood. Moreover, when dried blood was subjected to direct Carius digestion in Erlenmeyer flasks connected by ground glass joints to stoppered reflux condensers, failure to measure chloride completely was still experienced. These studies suggest that failure to measure the chloride completely in desiccated biological tissues must be attributed to other factors rather than to a loss of chloride.

We are in agreement with Norris and Ampt as to the desirability of alkaline digestion of certain biological materials before proceeding with the measurement of chloride. In the case of wet blood or serum, however, preliminary alkaline digestion was found unnecessary.

Our studies demonstrate that in some biological materials chloride is not completely measured by the direct open Carius method. Preliminary leaching with water in some instances provides almost complete recovery of the chloride; preliminary digestion with alkali is completely effective and is, therefore, the

procedure of choice in dealing with such materials. We have been unable to demonstrate that this failure to measure chloride by the direct open Carius method is due to a loss of chloride in amounts greater than 1·8 *mM* per litre of blood.

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CCXVII. ON MONOLAYERS OF PROTEOLYTIC ENZYMES AND PROTEINS.

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(*Received August 25th, 1933.*)

PART I.

THE REACTION KINETICS.

In a recent communication [Hughes and Rideal, 1933] it was shown that the progress of reactions taking place at an air-liquid interface could be followed by observation of the rate of change of the phase boundary potential. In this paper an account is given of a preliminary investigation of the action of a typical proteolytic enzyme system, trypsin on monolayers of egg-albumin and of caseinogen. This investigation was undertaken because it appeared probable that this type of reaction at a phase boundary frequently occurs in living systems.

The structure of albumin monolayers.

There is a considerable amount of information which indicates that the structure of proteins in bulk solution is different from that which exists when the protein is extended as a monolayer in a film.

The measurements of Svedberg and Nichols [1926] lead us to regard egg-albumin as an isodisperse protein of molecular weight 34,500, and from the constancy of its "molecular" weight it is assumed to be approximately spherical in form [*cf.* Sørensen, 1915]. The spherical character of the egg-albumin molecule is further supported by an optical examination due to Ettisch *et al.* [1931] who found that egg-albumin solutions in flow exhibit no birefringence and light scattered in the Tyndal beam is polarised. These views are opposed to those of Staudinger who regards eucolloids as existing in the filamentous or distended state in solution. The ease with which the protein particle breaks down outside the region of its stability limits, p_H 4 to p_H 9, suggests that the particle may not be really isodisperse within the limits but may consist of three units each of true molecular weight 11,500, built up into a single net-like unit (the choice of three similar units is based upon the approximate analysis by Vickery and Shore [1932]). A net-like structure has indeed been proposed by Kuhn [1932] for gelatin from an analysis of the viscosities of dilute solutions of this protein. It is clear that whichever view of the structure of egg-albumin in solution be correct, the balance of evidence is against the rod-like or filamentous structure. It has been shown [Hughes *et al.*, 1932; Fosbinder and Lessig, 1933] that a number of proteins may be spread directly as homogeneous surface monolayers from the solid material. Protein monolayers are characterised by the fact that under suitable conditions they form highly elastic gel-like films. For the polydisperse proteins such as gliadin and caseinogen in which the polypeptide chains are probably highly branched the gel state is not acquired until relatively high two-

dimensional pressures (15 dynes/cm.) are applied to the film; this phenomenon is accentuated in films of polypeptide mixtures such as peptone where solid films are formed only under very high compression. In the case of the isodisperse proteins like albumin the characteristic gel-like appearance is quite noticeable at relatively low pressures (< 1 dyne per cm.) and even below these pressures the films cannot be regarded as anything except weak gels developing into more homogeneous gels. If egg-albumin be spread on buffer solution it is found that the rate of spreading and the quantity per cm.^{2} attained at equilibrium suddenly increase several 100 % when the acidity of the substrate is extended beyond the stability region of the isodisperse state; it may be significant that this change coincides with the region of peptic digestion of the film.

Monolayers of the isodisperse and polydisperse proteins are also differentiated by the fact that in the polydisperse proteins such as gliadin and caseinogen, possessing many branched chains, the phase boundary potentials are relatively high whilst for the monodisperse proteins such as albumin they are much lower. The examination of the effect of changing the p_H of the substrate on the phase boundary potential likewise indicates that this is governed by the number and nature of the polar groups in the side-chains and is scarcely affected by the less polar amide linkages in the main polypeptide chain.

In the solid state Astbury and Wood's analysis [1932] has shown that the proteins exist in two characteristic forms—the zig-zag type of extended polypeptide chain, the β-keratin structure, and a chain folded in a series of hexagons of periodicity 5·1 Å., the α-keratin or diketopiperazine ring type. Whilst in the surface soluble proteins spreading out into monolayers the chains must be of the original β-keratin type, it seems certain that conversion into the α-keratin or diketopiperazine ring structure takes place in the surface and very rapidly, and it is to this latter structure that the stability of the films is due. Whilst the measurements of the limiting surface concentrations at the stage when gel formation commences are not sufficiently well defined experimentally to confirm this view as to their structure, yet the values obtained for the isodisperse proteins where the branch chains are not so prominent certainly favour this hypothesis. The following experimental observations may be cited in support of this view. The decrease in solubility of natural proteins after being converted into a film is well known and has been examined in detail by many investigators, notably Ramsden. Monolayers of egg-albumin may be heated to 85° and quickly cooled without apparent alteration in their physical properties. Natural albumin is readily dispersed by caustic soda more concentrated than p_H 11 but monolayers of albumin are very resistant even to $N/10$ caustic soda. On allowing even dilute solutions of proteins to stand [*cf.* du Noüy, 1926] the micelle in solution comes up to the surface and there spreads, undergoing the process of denaturation and loss of solubility. Finally, albumin in the film state is readily attacked by enzymes, a property not associated with natural albumin in bulk which appears to be very resistant, and this ease of attack is associated with the presence of diketopiperazine and anhydride rings in the molecule [Matsui, 1933]. It might be suggested that the process of denaturation at interfaces is associated with or may even consist in the conversion of the β-keratin or zig-zag structure into the α-keratin or diketopiperazine ring form.

We have noted that the examination of the phase boundary potential of a number of protein monolayers suggested that the main contribution to the phase boundary potential was from the polar groups in the side-chains and that the —CO—NH— linkage of the main chain contributed but little. On hydrolysis, if only one or more of these links be broken to form shorter polypeptide

chains terminating in —COOH and —NH_2 respectively, the phase boundary potential should increase until the peptide chain becomes so small that dissolution commences, and only then will the phase boundary potential commence to fall. As an alternative method, hydrolysis consists in the breaking either of an amide linkage near the end of a chain or two amide linkages close together in a chain and the small fragment containing relatively many polar groups thus released goes into solution. Under these conditions there will be a gradual fall in the phase boundary potential as the protein passes through the stages of peptone, polypeptide, tri- and di-peptide and simple amino-acid disintegration. As will be noted, this latter change is the one found experimentally and it is indeed possible to express, in a qualitative manner at least, the stages of disintegration of the protein by observation of the magnitude of the phase boundary potential.

Since the concentration necessary to form films is of the order of 10^{-7} g. per cm.^{2}, the method of surface potentials is not only particularly well suited for the characterisation and control of purity of biological materials which possess large surface potentials and are available only in small quantities but also in its sensitivity in measurement of chemical reactivity and rates of reaction for substances in almost sub-analytical quantities. In the case of these enzyme reactions in particular the kinetics can be followed over a convenient period of some twenty minutes at room temperature, and, furthermore, some light is shed on the actual nature of the enzymes themselves.

Thanks to the investigations of Willstätter and Grassman, Waldschmidt-Leitz and his co-workers and Northrop[1], we can distinguish in commercial trypsin solutions digesting egg-albumin at p_H 8–9, a proteinase, tryptase, effecting a primary hydrolysis into polypeptides; further hydrolysis proceeds by attack at the terminal groups in the polypeptide chain by polypeptidases. We may note that both the amino- and carboxy-polypeptidases which may be present in trypsin hydrolyse the amide linkages close to a terminal —NH_2 or —COOH group respectively with the result that small soluble fragments, *e.g.* leucine or tyrosine, are removed from the ends of the polypeptide chains, a point, as we have noted, of some importance in interpreting the effect of hydrolysis on the phase boundary potential. Whether a small soluble fragment is liberated in the action of the primary proteinase, tryptase, on the original protein has not been established but, as we shall note, such a suggestion is supported by the observed change in phase boundary potential. Still further hydrolysis is effected by the dipeptidases present.

Experimental procedure.

The experimental method employed followed closely that described in previous communications [Schulman and Rideal, 1931; Schulman and Hughes, 1932], with the exception that the apparatus was kept at 25°, the egg-albumin being spread from the solid from a micro-balance on the surface of $M/20$ buffer solutions made up according to the directions of Clark ("Determination of hydrogen ions") over a range of p_H 2–12; the solution was contained in a quartz trough of 300 cc. capacity. It was found necessary to take precautions to eliminate the formation of diffusion potentials in the liquid junctions connecting the buffer solution in the trough with the auxiliary electrode and membranes on the electrode itself.

The protein film so spread could be compressed by means of glass barriers to

[1] Compare *Ergebnisse der Enzymforschung*, Band 1, Leipzig, 1932.

the stage where the phase boundary potential on gelation no longer varied appreciably on further contraction; the film in this state possesses a characteristic surface potential dependent only on the p_H of the substrate. The water surface behind the barrier which had been moved to compress the film was left clean. 20 cc. of a suitable solution of the enzyme having stood at room temperature for half an hour in the same buffer solution as that on which the protein film was spread, but $M/10$ in strength (strong buffers being found necessary owing to the acidity of the trypsin), were injected and well mixed from the free side of the barrier into the underlying solution. With the aid of the moving polonium electrode the process of subsequent digestion of the protein film as well as the rate of adsorption of the ferment at the clean surface could be followed.

Owing to the high adsorbability of albumin at the surface of aqueous solutions of albumin, one part in half a million parts forming solid gel films at an air-liquid interface, it was found essential to eliminate soluble proteins from the enzyme. If the soluble protein is not eliminated it forms partially digested protein films at the surface which obscure and inhibit the main reaction so that the reaction appears to proceed in steps or jumps, the dimensions of which are dependent on the time the enzyme has been in solution before being injected into the trough. It was found that on standing at room temperature for half an hour the minute quantities remaining from a Merck's pancreatin were removed presumably by autodigestion. Solutions of pancreatin prepared in this manner were found to possess definite and reproducible phase boundary potentials, which could be employed as a criterion for their freedom from protein. All experiments were carried out with Merck's pancreatin, which gave more uniform results than other varieties.

The change in phase boundary potential due to an albumin film in the gel state on the buffer solution at p_H 7·7 is $\Delta V = 230$ mv. On injection of the pancreatin solution a steady decrease in the phase boundary potential is observed and the film loses its rigidity when the surface potential attains a value of *ca.* $\Delta V = 180$ mv., finally becoming quite fluid. The phase boundary potential finally attains a constant value of $\Delta V = 118$ mv. This value $\Delta V = 118$ mv. is identical with the characteristic value shown by a solution of pancreatin at this p_H so it appears that the protein film has been completely digested. On the protein-free side of the barrier within two minutes of injection of the trypsin solution a potential change of $\Delta V = 118$ mv. is obtained; the capillary active constituents in the pancreatin thus form a vaporous film of characteristic surface potential whose value does not change even on relatively rapid compression or expansion of the surface, a behaviour similar to that of substances which are capillary active and on solution in water form a Gibbs layer which may be regarded as a two-dimensional vapour or gas.

During the reaction the original protein gradually disappears from the film and we may assume that the removal of fragments of the protein from the surface is reflected in the change of phase boundary potential; this assumption is borne out by the fact that identical velocities of reaction are obtained when, instead of measuring the rate of change of phase boundary potential with time, maintaining the area of film constant, the rate of contraction of area of film necessary to maintain a constant phase boundary potential is measured. Experimentally, however, this latter procedure is difficult to carry out, especially in the later stages of digestion.

In Fig. 1 are shown the effects of variations in the concentration of pancreatin and of temperature on the rate of digestion of an albumin film at p_H 7·7.

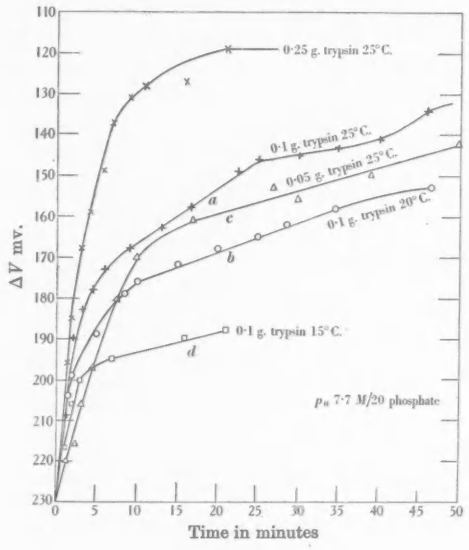

Fig. 1.

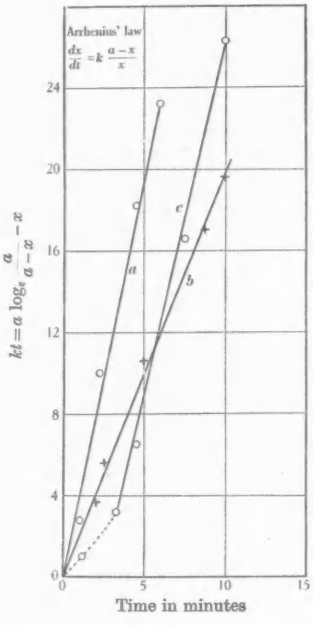

Fig. 2.

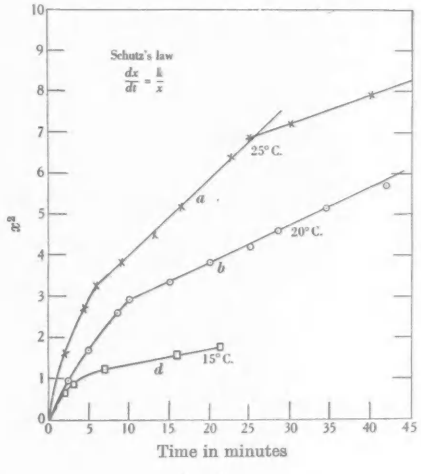

Fig. 2 *a*.

It will be noted from Figs. 2 and 2*a* that the reaction in presence of pancreatin, originally unimolecular, becomes converted on decreasing the concentration of pancreatin and at lower temperatures firstly into an inhibited reaction obeying the Arrhenius expression $\frac{dx}{dt} = k\frac{a-x}{x}$ (Fig. 2), then the rate of displacement of the by-products of the digestion by the trypsin alone governs the reaction velocity and the reaction follows Schutz's law $\frac{dx}{dt} = \frac{k}{x}$ (Fig. 2 *a*). Curves *a*, *b*, *c* and *d* are given as examples for the two expressions, *c* showing irregularity owing to diffusion to the surface of a weak trypsin solution.

A comparison of the actual velocity constants obtained for the digestion of films of caseinogen with the approximate values obtained for the reaction in bulk phase under similar conditions indicates that they are of the same order of magnitude, the chief difference is that the transition from a unimolecular surface reaction velocity to an inhibited reaction following Schutz's law takes place as a result of a smaller change in the concentration of the pancreatin; this is doubtless due to the greater facility with which by-products from the reaction can diffuse away from the disperse micelle in the bulk phase and the decreasing ability of the trypsin in the lower concentration to displace the by-products formed initially on the surface, since on complete digestion the pancreatin ultimately displaces all the by-products of digestion, as indicated by the attainment of identical values of ΔV_∞ on both sides of the barrier.

In Fig. 3 is plotted the logarithm of the change in phase boundary potential with the time and in Fig. 4 the unimolecular velocity constants respectively as a function of the p_H over the range of activity of the pancreatin. It will be noted that the surface reaction follows very closely that of the bulk reaction revealing a marked optimum between p_H 8 and p_H 8·4.

In the neighbourhood of the optimum the reaction only reveals a unimolecular reaction constant over the first few minutes, by-products being formed so rapidly that inhibition of the reaction sets in after a short period; thus the reaction as a whole proceeds more rapidly at p_H 7·5 than at p_H 8·2.

It is somewhat remarkable that the reaction at p_H 7·5, in spite of the complexity of the process, appears to be uniformly unimolecular over the whole course of the reaction, suggesting that the slowest of the enzyme reactions is that involving the first breakdown by the proteinase. Another noticeable feature of these reactions in films is that occasionally (approximately one experiment in every twenty) a period of induction was noted before the reaction commenced with its normal velocity, the usual phenomenon met with in the tryptic digestion of albumin when studied in bulk phase.

The specific proteolytic enzymes.

In order to examine the mechanism of tryptic hydrolysis in more detail, a number of experiments were carried out with proteinase and carboxypolypeptidase. We are indebted to Prof. J. H. Northrop of the Rockefeller Institute at Princeton for a sample of proteinase or crystalline trypsin (N) prepared by ammonium sulphate precipitation from pancreatin and also to D. R. P. Murray of the Sir William Dunn Biochemical Laboratory at Cambridge for a sample of proteinase (M) and of carboxypolypeptidase (P) prepared according to the method of Waldschmidt-Leitz by preferential adsorption on alumina.

In contrast with solutions of pancreatin, which at p_H 7·7 form only vaporous films with very low phase boundary potentials, these specific proteolytic ferments form solid gel-like films, possessing characteristics very similar to films of egg-

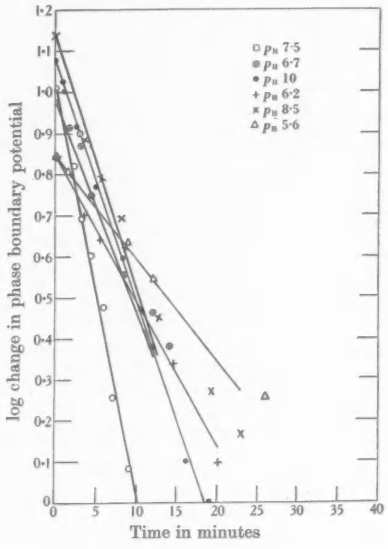
log change in phase boundary potential
Time in minutes
□ p_H 7·5
⊞ p_H 6·7
• p_H 10
+ p_H 6·2
× p_H 8·5
△ p_H 5·6

Fig. 3.

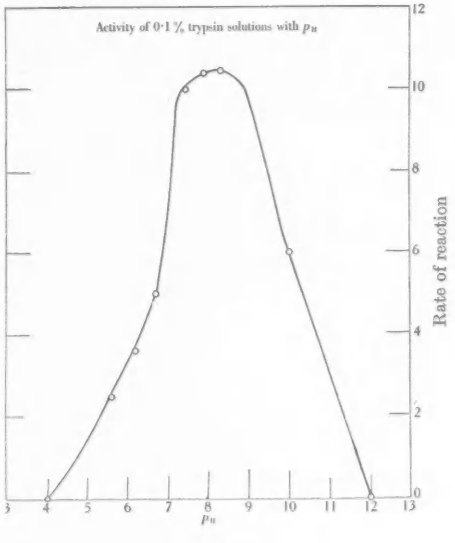
Activity of 0·1 % trypsin solutions with p_H
Rate of reaction
p_H

Fig. 4.

albumin, the phase boundary potential of the proteinases being $\Delta V = 220$ mv. and of the carboxypolypeptidases $\Delta V = 200$ mv. The proteinase N formed solid films very rapidly after injection of the solution into the buffer solution in the trough but some ten and twenty minutes respectively elapsed before the proteinase M and the carboxypolypeptidase P yielded solid films.

Solutions of these proteases were injected at p_H 7·7 under films both of egg-albumin and of caseinogen. In the examination of the stages of proteolytic digestion in films, caseinogen being polydisperse possesses certain advantages over egg-albumin in that not only is the change in phase boundary potential greater than for albumin in the region of gelation, but, accompanying a high surface pressure the region of gelation is smaller and more sensitive to changes in pressure.

In Figs. 5 and 6 are given a summary of the experimental results of the action of these enzymes on the two proteins.

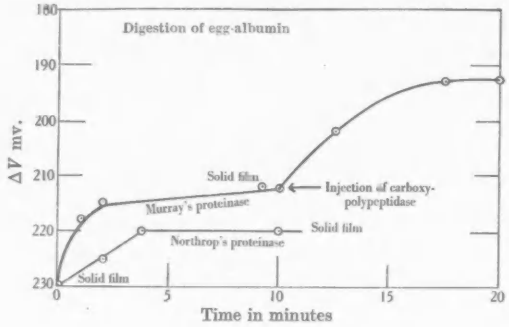

Fig. 5.

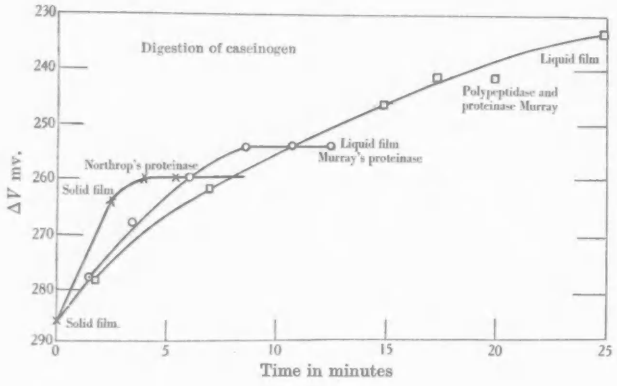

Fig. 6.

It will be observed that the proteinases N and M reduce the surface potential to a definite value, M, to a slightly greater extent than N, a fact probably due to the difficulty in elimination of the last traces of polypeptidases by the adsorption

process. Owing to the fact that only small quantities were available it has been impossible to determine the reaction constants, but, with the exception of the digestion of egg-albumin by Northrop's proteinase which follows a zero order, they appear to be unimolecular. In one case the carboxypolypeptidase P was injected into the solution of proteinase which had already digested a film of egg-albumin to the first stage. This resulted in a further stage of digestion with a concomitant fall in the surface potential of a further 20 mv. Likewise a mixture of M and P enzymes digested a caseinogen film with a change of 55 mv. compared with 25 mv. with the proteinase alone.

On comparison of these values with those obtained in the digestion of either egg-albumin or of caseinogen by pancreatin containing the complete series of enzymes, it is found that in the proteolytic digestion by the proteinase N about 10 % of the original protein has left the surface in some soluble form, whilst a further 25 % leaves the surface through the addition of the carboxypolypeptidase P.

On the other hand, about 15 % and a further 16 % leave the surface in the action of the ferments N and P respectively in the case of caseinogen. Another interesting feature observed in the digestion of a film of caseinogen, which possesses a region of gelation very sensitive to pressure (the rigidity of the gel being imperceptible when the phase boundary potential falls below 260 mv.), is that it is still in a gelatinous state when digested by the proteinase N, but on digestion by the ferment M which contained a slight trace of a carboxypolypeptidase a small further digestion reflected by a change of only 4 mv. in the phase boundary potential caused it to assume a fluid condition. The proteinase, however, rising to the free surface of the water on the other side of the glass barrier, forms a solid film. These conditions on each side could be maintained for several hours. The proteinase which itself forms a solid film does not displace the liquid film of the partly digested caseinogen owing to the higher surface pressure of the latter, and continuous gel-like films of proteins do not appear to be formed except at an air-liquid interface, no formation taking place underneath a monolayer.

PART II.

SOME STRUCTURAL CHARACTERISTICS OF THE ENZYMES.

The phase boundary potential of trypsin.

In Fig. 7 are plotted the phase boundary potentials of an albumin film in the gel state with that of a solution of trypsin for comparison as a function of the p_H of the medium.

It is interesting to note that at p_H 4 trypsin causes a marked alteration in the phase boundary potential, *viz.* $\Delta V = 320$ mv.; no tryptic activity is noted here, the film is gel-like and possesses all the properties of an albumin protein film. As we pass into the region of proteolytic activity the phase boundary potential falls, the surface loses its gel-like character and becomes perfectly fluid and similar to that of a vaporous or gaseous two-dimensional phase. Pepsin exhibits a similar behaviour, forming an albumin-like film at p_H 7 but a fairly mobile surface with a low phase boundary potential at p_H 2, the peptic optimum. The experimental evidence suggests that the trypsin solutions, even when prepared with the precautions stated, still contain small quantities of protein which form films at p_H 4; but at p_H 8 the rate of arrival of the protein micelle from the interior with

the subsequent spreading is equalled by the rate of tryptic digestion of the protein. In the region p_H 8 to 12 the phase boundary potentials are very similar as well as the fluidities of the surface, but at p_H 12 no surface tryptic activity is noted. It is probable that the traces of protein left in the solution of the enzyme in the micellar form are completely dispersed at this p_H and thus cannot form surface films by spreading from micelles. With strong solutions of trypsin or on

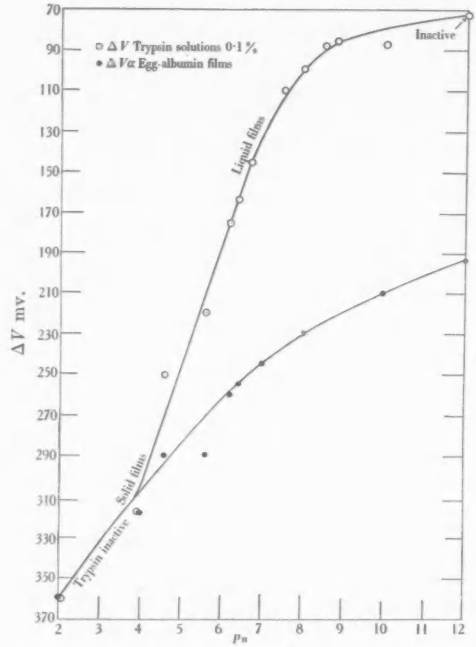

Fig. 7.

the addition of extra albumin solid gel-like films are eventually formed. Albumin when spread from a solid on to the surface forms a perfectly stable gel-like film at this p_H. It is clear that the characteristic surface potential which remains almost constant over this range of p_H, whilst no criterion for the activity of the ferment, is an indication that a distinct change has taken place in respect to the protein constituents in the surface solution.

That the films formed by pancreatin on acid substrates really consist of protein-like material was confirmed by the fact that they are readily digested by pepsin injected into the solution at p_H 2 in the usual manner.

The resultant active surface of pancreatin at the optimum p_H evidently contains no protein, although at p_H 2 undigested pancreatin forms films with all the characteristics of protein.

Similar digestion experiments were carried out with pepsin which forms protein-like films on alkaline solutions (p_H 7·7) of slightly lower $\Delta V = 220$ mv. compared with 240 mv. for albumin. These could be digested readily by pan-

creatin. Likewise the protein-like films formed by the proteinases obtained from Prof. Northrop and Dr Murray (see Part I) as well as by the carboxypolypeptidases could be digested in a similar manner by further injection of pancreatin.

Since the protein-like proteinase and the more disperse protein-like carboxypolypeptidase are further digested by addition of the lower trypsins, it would suggest that the various tryptic ferments are associated with their own breakdown product of original protein when in solution at the p_H for optimum activity, and over the inactive region of p_H with proteins albumin-like in character.

It is clear that all these enzyme preparations are associated with the presence of protein-like material which is capable of being digested by other enzymes or by autodigestion and, also, after removal of the protein by digestion it appears that the surface enzymic activity of the solutions remains unimpaired.

The conclusions inferred from these experiments could logically be extended to enzyme solutions which have been digested by other enzymes, *e.g.* pancreatin by pepsin. These have already been examined by Northrop who showed that the enzymic activity in solution decreased as the digestion of the proteinase proceeded. We have found by digesting pancreatin with pepsin at p_H 2 and then bringing the solution back to p_H 7·7 that in agreement with Northrop's observation the tryptic activity in bulk disappears as the peptic digestion becomes more complete. On the other hand the very interesting observation was made that whereas these solutions were inactive for the digestion of caseinogen, albumin or gelatin in the bulk phase, if films were formed of either of the former proteins on these apparently inert solutions, digestion took place rapidly with unimpaired activity.

The method consisted in dissolving 0·3 g. pancreatin and 0·2 g. pepsin in 50 cc. $N/100$ HCl, leaving to digest for varying lengths of time at 38° and injecting the mixture into the trough containing 250 cc. phosphate buffer at p_H 7·4. The changes in films of egg-albumin or caseinogen spread upon the surface were then observed. It was noticeable that on prolonged digestion of the pancreatin by pepsin the surface proteolytic activity became greater until it reached the normal activity of undigested pancreatin after 24 hours' treatment. This was probably due to the removal from the solution by peptic digestion of protein which inhibits the surface reaction. Even after 48 hours' digestion by pepsin a considerable surface tryptic activity was observed. A control experiment indicated that the tryptic activity of pancreatin appeared to be unimpaired when it was dissolved in 50 cc. $N/100$ HCl and the solution subsequently injected into a phosphate buffer at p_H 7·4.

In the curves in Fig. 8 are shown the results obtained and it is seen that this activity for surface digestion is maintained for relatively long periods of time; whilst after only 5 hours' digestion of pancreatin by pepsin the activity in the bulk phase is scarcely measurable when tested with caseinogen and precipitation by acetic acid. Pepsin, on the other hand, is quite inactive on protein films at p_H 7·7 and when the acidity is brought back to p_H 2 it is still completely inactive; this is analogous to its behaviour in the bulk phase.

From the curves in Fig. 1 (Part I) it is seen that the surface activity is very sensitive to changes in the bulk concentration of pancreatin, and we must conclude that in these experiments the concentration of active enzymes in the bulk phase has been left unaltered by the peptic digestion, although the solutions exhibit no tryptic activity in the bulk phase.

Two points emerge from these film experiments; firstly, that the active enzyme can be removed from its protein-like carrier by suitable digestion. This appears to be in agreement with the experiment of Dyckerhoff and Tewes [1933],

who effected an exchange of protein carrier for the pepsin, making edestin and other protein carriers peptically active by precipitation from pepsin solutions, whilst Frenkel [1932] effected a separation of a toxin from its protein carrier, leaving an active solution containing no protein in the case of diphtheria toxin.

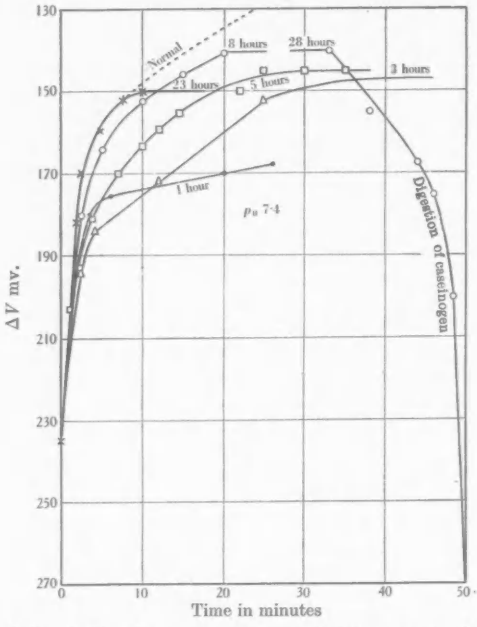

Fig. 8. Digestion of albumin films by trypsin solutions which have previously been digested by pepsin at p_H 2. 0·2 g. pepsin + 0·3 g. trypsin in 50 cc. HCl; in bulk, no reaction after 5 hours.

The second point of interest is that solutions of these enzymes from which their normal protein-like carriers have been removed by digestion are incapable of exerting any enzymic activity on proteins except when these latter are presented to them in the particular state or orientation associated with the film. We have so far been unsuccessful in effecting a regeneration of bulk enzyme activity by the addition of a suitable support material, although we have included both inactive enterokinase and edestin, as well as albumin denatured in various ways, including that denatured by film formation, and also substances such as alumina and quartz. The experimental investigation is being continued in this direction.

It has long been known that destruction of enzymic activity in respect to its activity in a bulk phase proceeds rapidly on boiling. A series of experiments was carried out with a view to investigating the existence (in boiled solutions) of any residual enzyme activity to protein monolayers. Pancreatin solutions containing 0·3 g. in 20 cc. of buffer solution were boiled for various periods of time and cooled rapidly to 0° and then injected into the trough containing the buffer solutions and their surface enzymic activity measured by the rate of hydrolysis of either albumin or caseinogen films; comparative runs with unboiled solutions were carried out at the same time. The results obtained are shown in Figs. 9 and 10.

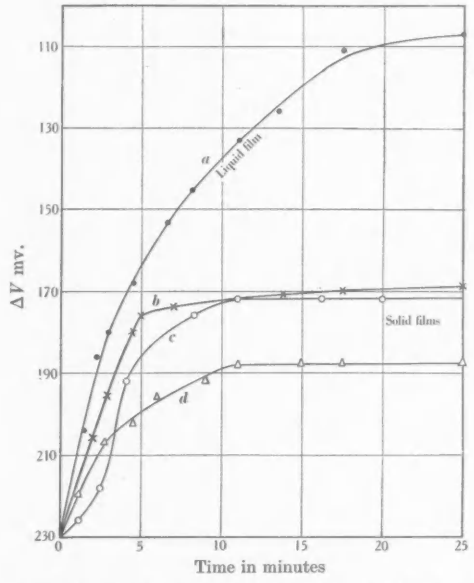

Fig. 9. Digestion of egg-albumin by boiled trypsin solutions p_H 7·8. *a*. Normal 0·1 % trypsin solution. *b*. Solution heated for 5 minutes. *c*. Solution heated for 30 minutes. *d*. Solution just brought to boiling.

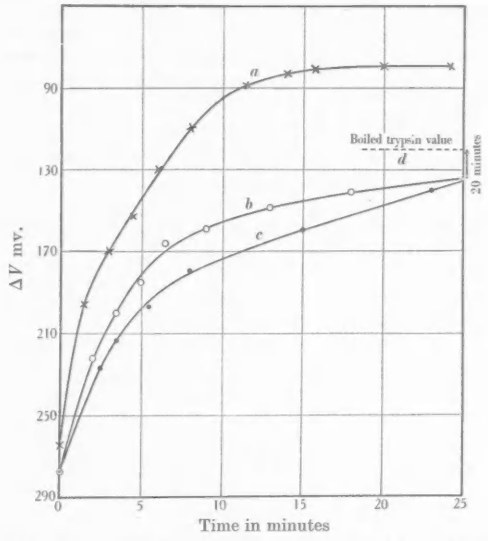

Fig. 10. Digestion of caseinogen by boiled trypsin solutions p_H 8·2. *a*. Normal. *b*. Heated to boiling at p_H 8·2. *c*. Boiled for 30 minutes at p_H 8·2. *d*. Boiled for 30 minutes at p_H 4·6 and filtered, no reaction.

It will be observed that solutions of trypsin when boiled at p_H 8 for a period even as long as 30 minutes still exert a marked tryptic activity on protein films, the activity being still about one-third that of the original solution, whilst no activity could be noticed in bulk as tested by caseinogen and acetic acid precipitation. It may be remarked that egg-albumin films appear to be more resistant to the boiled enzyme than films of caseinogen, which liquefy and disappear in the normal manner. The ultimate phase boundary potentials obtained are some 40 mv. higher for the boiled than for the unboiled solutions, showing that some change has occurred in the component parts of the pancreatin.

The results of these experiments carried out at p_H 8 are to be contrasted with the results obtained by boiling a trypsin solution at p_H 4·6, filtering off the coagulated protein and bringing the p_H back to 8 again. These solutions exhibit no tryptic activity either in bulk or for protein films and indeed form solid protein-like films themselves at an air-liquid interface which, however, in no way affect the solidity of a gelatinous caseinogen film if a solution of the boiled enzyme be injected underneath. It appears that the enzyme surface activity is in direct relation to the degree of coagulation and consequent insolubility of the protein carrier; for at p_H 8 there is only a slight precipitation of coagulated protein and a lengthening of the digestion period of a film from some fifteen to forty-five minutes.

It might be concluded from these experiments that the enzyme consists of at least two components, a "free enzyme" non-protein in character and a protein possessing a specific structure, generally adsorbing or reversibly binding the free enzyme. The "free enzyme" readily reacts with proteins when presented to it in the form of an orientated protein monolayer but does not react with proteins in solution. To impart activity for a bulk phase reaction to the "free ferment" the latter must be adsorbed on a protein possessing a specific structure, for although it is readily adsorbable by coagulated proteins it is inactive both for surface and bulk reaction in this state. It is possible that the function of active enterokinase is to present such a surface.

It may be supposed that an enzyme consists of a system which includes several separate components, the "free enzyme," which in the case of the proteolytic enzymes appears to be non-protein in character, the free enzyme carrier which is specific and protein-like in character and behaves in a manner similar to that of an orientated monolayer and of surface activators such as enterokinase. The activity of the complex of free enzyme and its carrier is dependent on its environment both in respect to its power of adsorption and its chemical reactivity. It is hoped that further experiments on the relationship between bulk and surface reactivity will throw further light on the characteristics of the enzyme system.

It is interesting to note here that when these proteolytic ferments are treated chemically, *i.e.* with acids or alkalis or by poisoning with metallic ions such as Ag^+ or Cu^{++}, their activity is destroyed equally both for bulk and surface reactions; on the other hand, when they are treated by boiling or the protein removed by digestion by another enzyme, only the bulk activity is impaired.

The interpretation advanced for these experiments revealing the peculiar relationship existing between the activity of what has been designated the "free enzyme" and proteins when presented to the "free enzyme" in the form of an orientated monolayer is based upon the hypothesis that the changes in phase boundary potential observed when a solution of an enzyme is injected under the surface of a buffer solution covered with a protein monolayer can be taken as a measure of enzymic activity, *i.e.* a digestion of protein by the enzyme. In

support of this hypothesis may be advanced the results of the investigations described in Part I where the process of gradual liquefaction of a protein film by an enzyme was described, the coincidence of the p_H optima for both bulk and surface reactions, the fractional changes effected by the proteinase and carboxypolypeptidase respectively, and lastly the various forms of what have been termed velocity curves obtained with different concentrations of enzyme, which curves are faithful replicas of the velocity curves obtaining for bulk solution in these systems.

It seemed desirable to show more directly that the disappearance of a protein film from the surface of a buffer solution after injection into that solution of a proteolytic enzyme was in reality a digestion of the protein.

A solution containing both egg-albumin and pancreatin at p_H 8 was allowed to undergo digestion. From time to time a few drops of the solution were placed by means of a dropping pipette on the surface of a buffer solution at p_H 8 and the change in phase boundary potential noted. The phase boundary potential in all cases attained the characteristic value obtained for the original protein with the formation of a solid film until complete digestion had taken place as determined by the absence of a precipitate with acetic acid, when the value characteristic of solutions of trypsin would appear and no solid film be formed. Evidently undigested protein in the drops spreads on the surface in preference to the trypsin or the products of hydrolysis until no protein is left in the solution. The identity of the phase boundary potential with that of the protein reveals no evidence of a protein-trypsin complex, although, as will be noted in the next series of experiments, evidence for the existence of a complex between some constituent in the pancreatin and a breakdown product of egg-albumin can be presented.

In the next series the trough was filled with buffer solution containing pancreatin and egg-albumin in various concentration ratios and the phase boundary potential determined from time to time as the process of bulk digestion proceeded.

It was found that the phase boundary potentials shown by these solutions were very sensitive to the ratio of the pancreatin to albumin. When the original solution contains a slight excess of pancreatin to protein the surface of the solution is found to acquire a solid film rapidly with all the characteristics of the protein, but this liquefies in the manner described in the investigations on monolayers, and within a few minutes the phase boundary potential acquires the characteristic value of a pancreatin solution (Fig. 11, graph *a*).

In experiments in which equal proportions of pancreatin and egg-albumin are present (0·1 % of each in the solution), the surface rapidly acquires a solid protein film with the characteristic phase boundary potential for egg-albumin (graph *b*); this falls slowly as the concentration of the protein diminishes until what must be regarded as a complex of trypsin and a product of hydrolysis of the protein commences to penetrate the surface layer, gradually increasing in quantity as the protein diminishes until ultimately the surface consists entirely of the trypsin complex possessing phase boundary potentials dependent upon slight variations in the original ratio of enzyme to protein, in general much higher (*e.g.* 178 mv.) than that of a trypsin solution (90 mv. at p_H 8). The surface is, however, perfectly fluid and no change takes place in the phase boundary potential on contracting the surface area by means of a slide. In the last case where the solution contains an excess of protein to pancreatin the surface reaction evidently becomes more complicated (graph *c*). It is observed that the protein film of phase boundary potential 250 mv. forms immediately as in the other cases. The phase boundary potential falls in twenty minutes to 193 mv. and then com-

mences to rise again. The originally rigid gel-like film decreases in rigidity, becomes more elastic and then eventually becomes rigid again. At the end of 220 minutes the original protein-like characteristics of the surface film have all returned.

These films are, however, ultimately digested away after prolonged periods, *ca.* 10–15 hours.

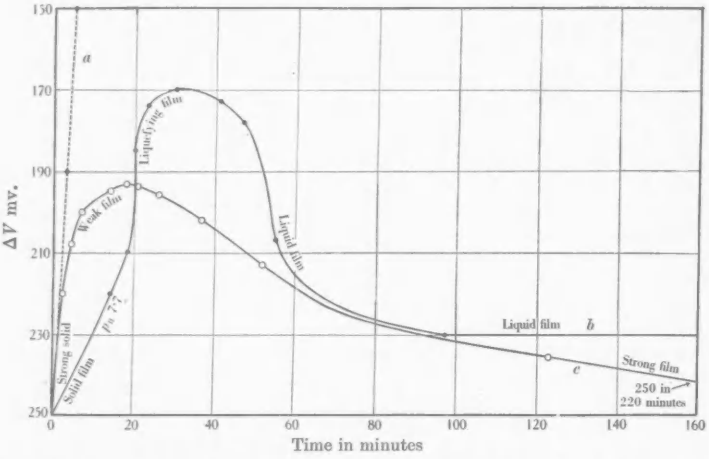

Fig. 11. Surface potential of bulk phosphate at p_H 7·7. *a*. 0·3 g. trypsin + 0·2 g. egg-albumin in 300 cc. at 25°. *b*. 0·3 g. trypsin + 0·3 g. egg-albumin in 300 cc. at 25°. *c*. 0·1 g. trypsin + 0·2 g. egg-albumin in 300 cc. at 25°.

The simplest interpretation to be placed on these observations is that after a preliminary partial digestion of the surface film has taken place the products and the enzyme present near the surface are displaced by the large quantities of undigested protein in the bulk phase and the regenerated protein film then persists until the concentration of the protein in the bulk phase has been greatly reduced.

Further experiments were carried out to test the effects of known by-products in the pancreatin (lipase and amylase) on a protein monolayer.

A pancreatin solution was filtered at p_H 4·6 and the filtrate treated with alumina B. The elution from the alumina containing the lipase and amylase did not influence the surface potential of a caseinogen film, whereas the residue containing the tryptic ferments displaced the caseinogen film in the usual manner.

It may be concluded from these experiments that the change in phase boundary potential and disappearance of a protein film may be taken as an indication that proteolytic hydrolysis or digestion is in reality proceeding.

Summary.

It is shown that when solutions of pancreatin are injected under monolayers of proteins such as egg-albumin or caseinogen proteolytic hydrolysis of the latter proceeds. The reaction can be followed qualitatively visually and quantitatively by observing the concomitant changes in phase boundary potentials.

The reaction kinetics of the proteolytic digestion of protein monolayers follow closely those previously observed for the bulk phase reaction both in respect to dependence on the p_H of the solution and the order of the reaction. It is shown that the proteinase fraction of pancreatin is responsible for the removal of some 10 % of the protein in the case of egg-albumin and of 15 % in the case of caseinogen, whilst the carboxypolypeptidase fraction removes a further 25 and 16 % respectively.

The proteinase and carboxypolypeptidase fractions of pancreatin when purified by the precipitation or by the adsorption process form films which are protein-like in character. These are themselves capable of being hydrolysed by pancreatin without loss of surface activity.

The characteristics of films of trypsin are found to be indistinguishable from those of albumin over the region of p_H in which they exhibit no tryptic activity, whilst over the active region they form solutions of low surface phase boundary potentials.

It is shown that when pancreatin solutions are digested by pepsin at p_H 2 and the solution restored to p_H 8, whilst no tryptic activity is observed for bulk phase reaction, monolayers of egg-albumin and caseinogen are readily digested. Solutions of trypsin when boiled at p_H 8 likewise exhibit a marked surface proteolytic activity.

The properties of the surfaces of solutions containing both enzyme and protein are examined during the course of the reaction. It is concluded that in the tryptic enzymes the "free enzymes" can be separated from their protein-like carriers but when free can only react with proteins when presented to them in a suitable form such as a monolayer at an air-water interface. To render the "free enzyme" active for protein solutions it must be adsorbed on a protein in a particular state or configuration. Experimental evidence is given that the disappearance of a protein film on an enzyme containing substrate is due to digestion only and is not due to the adsorption of some constituent of the enzyme of lower surface tension.

We wish to express our thanks both to Prof. Northrop of Princeton and to D. R. P. Murray for their kindness in furnishing us with specimens of the purified enzymes and to the latter for assistance in carrying out bulk control experiments; to Dr J. Zozoya for much helpful criticism and to the Medical Research Council for a grant to one of us (J. S.).

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CCXVIII. FERMENTABLE SUGAR IN FASTING URINE.

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There seems no doubt that the post-prandial urine of normal men and women often contains a reducing substance or substances removable by yeast. This "fermentable sugar" has been found after breakfast by Van Slyke and Hawkins [1929], at irregular intervals during the day both by Greenwald *et al.* [1927] and by Harding and Selby [1931], and in 24 hour urines by West and Peterson [1932]. Its occurrence varies with the nature of the meal (Harding and Selby) or the general diet [West, Lange and Peterson, 1932] and with the individual (Harding and Selby). Whilst all the methods for its determination have in common the use of yeast, different reagents have been used for the determination of the reducing power, thus minimising the possibility that the findings are due to some extraneous accident of technique. The fraction removable by yeast almost certainly contains glucose. The fraction is found in the carbohydrates of the urine. Harding and Selby provided physiological evidence for the entry of glucose from the blood, and West and Steiner [1932] state that the rate of fermentation is almost exactly paralleled by the same amount of glucose under the same conditions. On the other hand Greenwald *et al.* believed it to be non-glucose in character, and Van Slyke and Hawkins have clearly shown the simultaneous occurrence of small amounts of irregularly excreted non-glucose substance removable by yeast. Harding and Selby also showed the possible occurrence of fructose.

The previous paper of Harding and Selby reported the practical absence of a substance removable by yeast from fasting urines and at many other times during the 24 hours. We did not deny the total absence of glucose, but recognising the limitations of the method of analysis, regarded such amounts of that sugar as might be present in these urines as minimum. Certainly there was no difficulty in differentiating between these possible minimum amounts and the comparatively large excretion of post-prandial substances removable by yeast. This irregularity of excretion, affecting both the time of day and the individual, enabled the authors to harmonise their own results with the previous contradictory findings reported in the literature.

In a recent series of papers West and his colleagues [West and Peterson, 1932; West, Lange and Peterson, 1932; West and Steiner, 1932] claim that fermentable sugar occurs in all urines. This they identify as glucose.

The crucial point of difference between ourselves and West *et al.* lies in the possible amount of fermentable sugar in fasting urine. West and Peterson ascribe our lack of ability to find fermentable sugar in fasting urines to the operation of a number of causes which, acting together, reduce the effective amount of this substance to such an extent that the titration difference between the total sugar and non-fermentable sugar becomes indistinguishable from the

titration error. They calculate that the presence of 6 mg./100 cc. fermentable sugar in urine at a dilution 1 : 10 would only mean a titration difference of 0·08 cc. 0·005*N* thiosulphate under our conditions, and a difference of 0·04 cc. at dilution 1 : 20. West, Lange and Peterson go so far as to remark "that the method used by these workers is incapable of estimating with sufficient accuracy the fermentable sugar of starvation urines."

In our previous paper we drew the conclusion that "the possible amounts of fermentable sugar in normal fasting urine must be under 5 mg./100 cc. and are more likely to be less than 2–3 mg./100 cc." The results on which this conclusion is based are to be seen in Tables I and II of the previous paper. In Table I, where the dilution is constant at 1 : 10, only 1 urine out of 8 shows a titration difference of 0·04 cc. Evidently in this series of urines, the amount of fermentable sugar is 3 mg./100 cc. or less, and such a conclusion neglects the fact that some of the urines gained slightly in reducing power, rather than lost. In Table II the dilutions are varied, but only 4 out of 18 urines show fermentable sugar equal to 0·03 cc. 12 out of the 18 show titration differences of +0·01 or 0·00 cc. The average result lies within our experimental error, and we consider our conclusion justified. Our conclusions were supported also by an unpublished series of observations on 8 normal fasting urines of which we now give the details of 4. The methods were those of our previous paper.

Extreme care was taken in this series. All apparatus was recalibrated and especially cleaned before use. The yeast was most thoroughly washed and after use on the diluted urine was removed by a double centrifuging. Two samples from each urine specimen were analysed separately, with duplicate titrations on each. The extreme range of all 4 titrations did not exceed 0·02 cc. 0·005*N* thiosulphate. The average value thus only varies from the extreme by ±0·01. Table I shows the results of 2 urines diluted 1 : 10 and 2 diluted 1 : 20. The amount of sugar removable by yeast is represented by differences of ±0·01 cc.

Table I.

Subject	Specimen	Final dilution	Total sugar	Non-fermentable sugar	Fermentable sugar
			cc. 0·005*N* thiosulphate per 2·0 cc. filtrate		
I	1		1·02	1·03	
		1 : 10	1·02	1·02	
	2		1·01	1·03	
			1·02	1·01	
		Average	1·02	1·02	0·00
II	1		0·83	0·84	
		1 : 10	0·83	0·83	
	2		0·82	0·84	
			0·83	0·84	
		Average	0·83	0·84	−0·01
III	1		0·57	0·55	
		1 : 20	0·57	0·54	
	2		0·55	0·55	
			0·56	0·55	
		Average	0·56	0·55	+0·01
IV	1		0·66	0·64	
		1 : 20	0·64	0·65	
	2		0·65	0·65	
			0·64	0·64	
		Average	0·65	0·65	0·00

It would seem a justifiable conclusion that fermentable sugar, or sugar removable by yeast, was present in these urines only in minimum amounts. Had we been dealing with aqueous solutions of glucose we should have detected with certainty 1–2 mg./100 cc. With urines the sensitivity of our reagents appeared somewhat less, and we set the limits of detection at 2–3 mg./100 cc. Our reagents and methods were of sufficient delicacy to detect glucose in the concentrations stated by West *et al.*

So far neither West *et al.* nor ourselves have confirmed the presence in urine of the non-glucose reducing substance removable by yeast of Van Slyke and Hawkins. These latter obtained evidence for its existence in urines diluted only 1 : 1, when acted on by an equal volume of washed centrifuged yeast cells. There was possibly some indication of its existence in the urine No. 1 in Table I of our previous paper, where 3 successive treatments with yeast removed equivalents of 0·02, 0·03 and 0·03 cc. 0·005*N* thiosulphate. The amount was very small, so small that we felt no correction was required in the majority of urines. Nevertheless we minimised the possible interference of this substance by using a highly diluted urine. At the same time in our study of post-prandial urines we refused to consider as true fermentable sugar any difference less than 0·05 cc. 0·005*N* thiosulphate. The use of urines of only moderate dilution, and the interpretation of very small diminutions in reducing power after the action of yeast as true fermentable sugar, were open to the criticism that we were dealing with the Van Slyke-Hawkins or a similar substance, which when present in very small amounts became completely removable by a single yeast action.

Although as a result of West's criticism we have felt ourselves under the necessity of defending and amplifying our previous work, nevertheless it is a matter of surprise that there is any serious matter for disagreement. Both laboratories use the same general method of analysis, the yeast preparations and their use are the same, and the copper reagents differ only in a few details. Both methods quantitatively recover added glucose, either as a direct addition to the total sugar value, or as extra fermentable sugar. If the fermentable sugar found by West *et al.* in fasting human urine consists entirely of glucose, the two sets of observations should be in substantial, if not complete, agreement. True, West uses $HgSO_4$–$BaCO_3$ in his preliminary treatment of the urine and we have used H_2SO_4–Lloyd's reagent. This however should affect only the level of the total sugar and non-fermentable sugar determinations and not their difference, if glucose is the sole urinary fermentable sugar. The present paper contains the results of some observations directed towards discovering if possible the cause of the discrepancies.

General methods.

The general method of analysis is the same as in our previous paper. After dilution the urine is treated with a suitable reagent to remove interfering substances. The total "sugar" is determined on 2 cc. filtrate. Another portion of the diluted urine (10–15 cc.) is treated with 0·25 g. wet-weight washed centrifuged yeast for 8 minutes at 37° with constant stirring. The yeast is removed by centrifuge. The non-fermentable "sugar" is determined on 2 cc. of this centrifugate after clearing. All thiosulphate titrations were performed in triplicate. Two copper reagents have been used. The first is that described by Harding and Selby [1931] and the second is that described by Harding and Downs [1933]. The chief difference between them is the carbonate-bicarbonate ratio. We shall refer to these reagents as 1 and 2.

All urine-filtrates were adjusted to p_H 6·5 previous to "sugar" determinations.

Influence of ammonia on "sugar" determinations.

Harding and Downs [1933] showed that very small concentrations of ammonium salts decreased the sensitivity of their reagent to sugars in aqueous solution. The removal of ammonia could readily be effected by the addition of KH_2PO_4 followed by solid MgO. This gave a filtrate containing the original amount of sugar. The presence of ammonium salts in urines may thus cause a certain amount of error in sugar determinations, unless the preliminary "clearing" at the same time removes the ammonia. In normal urines the amount is not large, and the error, in view of the uncertainty of interpretation to be attached to total sugar determinations, is probably not serious. The use of $HgSO_4$–$BaCO_3$ as recommended by West gives a filtrate free from NH_3. Lloyd's reagent leaves some NH_3, but this clearing agent can be preceded or followed by the KH_2PO_4–MgO treatment.

However great or small may be this possible source of error on the determination of total sugar, within the limits of its normal variation, the presence of NH_3 has no effect on the yeast action. There thus appears to be no reason to suspect an inaccuracy of our previous determinations of fermentable sugar from this cause.

A fasting 2 hour urine sample was diluted to 150 cc. To 120 cc. of the diluted urine were added 1·2 g. KH_2PO_4 and 2·4 g. solid light calcined MgO. After shaking 15 minutes the mixture was filtered and the filtrate found NH_3-free by Nessler's reagent. The filtrate was brought to p_H 6·5 by adding 1 or 2 drops of 20 % H_2SO_4. 100 cc. of 0·04 *N* H_2SO_4 were added to 100 cc. urine-filtrate and 6 g. Lloyd's reagent. After shaking a few minutes, the treated urine was filtered. A determination of total sugar and non-fermentable sugar after yeast action was made. To 50 cc. of the cleared diluted urine were added 50 mg. $(NH_4)_2SO_4$. This represents approximately 150 mg. NH_3 per 100 cc. original urine. Total sugar and non-fermentable sugar after yeast action were again determined. The added NH_3 was removed from the remainder of the liquid by KH_2PO_4 and MgO. Total sugar and non-fermentable sugar after yeast action were again determined.

The sugar estimations were made by both copper reagents. The results, shown in Table II, demonstrate the very slight effect of the added ammonium salt when the estimations were made by reagent 1 and the marked effect on reagent 2. The effect, however, is the same on both total sugar and non-fermentable sugar determinations. The yeast action is evidently unaffected.

Table II. *Showing the action of added NH_3 on total sugar and non-fermentable sugar determinations and the accuracy of fermentable sugar determinations in presence of ammonium salts.*

	"Sugar" as cc. 0·005 *N* thiosulphate per 2 cc. filtrate					
	Reagent 1			Reagent 2		
Procedure on urine	T.S.	N.F.S.	F.S.	T.S.	N.F.S.	F.S.
KH_2PO_4–MgO and H_2SO_4–Lloyd's reagent	2·82	2·44	0·38	2·07	1·77	0·30
As above + added $(NH_4)_2SO_4$	2·80	2·42	0·38	1·75	1·45	0·30
Added $(NH_4)_2SO_4$ removed by KH_2PO_4–MgO	2·82	2·44	0·38	2·07	1·76	0·31

T.S. = Total sugar. N.F.S. = Non-fermentable sugar. F.S. = Fermentable sugar.

The use of KH_2PO_4–MgO supplies another method for "clearing" urines previous to sugar determinations. This method removes very little reducing material, giving high "sugar" values when used alone. Such urines are Ca-free and contain only a small amount of phosphate.

Effect of variations in methods of clearing of urine and different copper reagents.

If the directions of West and Peterson for the use of $HgSO_4$–$BaCO_3$ are followed and the interfering substances of urine removed by this reagent, there is no difficulty in finding fermentable or yeast-removable sugar in every fasting urine we have examined, at dilutions varying from 1 : 5 to 1 : 15 and using 2 cc. filtrate for the titrations. We have found it convenient to express our results in mg. glucose per 2 hours, but assuming a regular excretion throughout the day the amount would vary from 36 to 60 mg. The concentration varies from 4 to 15 mg./100 cc. The yeast was used on diluted urine and previous to the $HgSO_4$–$BaCO_3$ treatment.

If the diluted urine is treated with KH_2PO_4–MgO followed by H_2SO_4–Lloyd's reagent, following directions similar to those given in the previous section, and the yeast action is allowed to take place just previous to the treatment with H_2SO_4–Lloyd's reagent, a series of figures is obtained for fermentable sugar similar to those obtained by the use of $HgSO_4$–$BaCO_3$. Both results are independent of the copper reagent.

If the treatment or "clearing" of the urine is solely by H_2SO_4–Lloyd's reagent, two sets of results are obtained for fermentable sugar. The determination of total and non-fermentable sugar by reagent 1 (the reagent used in our previous paper) gives figures for fermentable sugar approximately one-half of those given by reagent 2, or by either reagent after the use of $HgSO_4$–$BaCO_3$ *etc.* The fermentable sugar is now 1 to 3 mg. per 2 hours, reckoned as glucose, and its concentration varies from 2 to 6 mg./100 cc.

All these results are shown in Table III. The values for fermentable sugar are obtained by subtraction of the non-fermentable sugar from the total sugar values in mg./2 hours and not by calculation from the difference as given in cc. 0·005N thiosulphate. This causes occasional slight discrepancies between

Table III. *Showing the effect of different clearing reagents on the estimation of total (T.S.), non-fermentable (N.F.S.) and fermentable (F.S.) sugar.*

Figures represent mg./2 hours as glucose. Bracketed figures are cc. 0·005N thiosulphate for 2 cc. urine filtrate.

Subject	Vol./ 2 hours cc.	Approx. final dilution	H_2SO_4–Lloyd's reagent T.S.	N.F.S.	F.S.	KH_2PO_4–MgO and H_2SO_4–Lloyd's reagent T.S.	N.F.S.	F.S.	$HgSO_4$–$BaCO_3$ T.S.	N.F.S.	F.S.	Copper reagent
A.R.A.	34	1 : 15	36	34	2 (0·06)	40	35	5 (0·13)	24	20	4 (0·11)	1
			28	24	4 (0·15)	28	23	5 (0·15)	21	16	5 (0·15)	2
T.F.N.	94	1 : 5	49	47	2 (0·05)	51	46	5 (0·15)	30	25	5 (0·14)	1
			32	28	4 (0·13)	35	30	5 (0·16)	25	20	5 (0·17)	2
D.L.S.	55	1 : 10	50	48	2 (0·04)	50	45	5 (0·12)	27	24	3 (0·09)	1
			31	29	2 (0·09)	33	30	3 (0·11)	23	19	4 (0·12)	2
G.A.G.	56	1 : 10	54	53	1 (0·03)	52	48	4 (0·13)	30	25	5 (0·14)	1
			37	33	4 (0·12)	37	33	4 (0·12)	26	21	5 (0·12)	2
S.J.	112	1 : 5	50	47	3 (0·07)	48	43	5 (0·14)	28	24	4 (0·10)	1
			32	28	4 (0·13)	33	28	5 (0·15)	28	24	4 (0·17)	2
C.E.D.	57	1 : 10	43	41	2 (0·06)	48	42	6 (0·18)	25	21	4 (0·10)	1
			27	22	5 (0·16)	31	26	5 (0·17)	20	16	4 (0·15)	2
C.E.D.	50	1 : 10	42	40	2 (0·07)	46	40	6 (0·17)	23	19	4 (0·10)	1
			30	26	4 (0·14)	33	28	5 (0·18)	21	17	4 (0·15)	2

the values as shown in the two columns, as we have approximated the values for total and non-fermentable sugar to the nearest mg.

Two questions at once arise. Why does copper reagent 1 compared with reagent 2, and used after H_2SO_4–Lloyd's reagent, estimate only one-half the fermentable sugar, and why do the results not agree with those of Tables I and II of our previous paper and Table I of the present paper? To the first question it would seem a reasonable answer that reagent 2 determines some non-glucose substance removable by yeast which is estimated either partially or not at all by reagent 1. If so, this substance might have been altered by the previous treatment of the urine by KH_2PO_4–MgO, for the fermentable sugar is now the same by both reagents. Treatment of urine with $HgSO_4$–$BaCO_3$ apparently also alters its value to the same degree, unless the agreement is entirely fortuitous. It must be remembered that the yeast action in our experiments has taken place before the treatment with H_2SO_4–Lloyd's reagent or $HgSO_4$–$BaCO_3$, so that any change should be shown as an apparent increase in total sugar. Of the two copper reagents No. 2 was designed to have a very much smaller oxidising power on some nitrogenous reducing substances, and $HgSO_4$–$BaCO_3$ was designed by West to remove a number of such possible substances. Changes of this character overshadow to such an extent any possible alterations in the reducing power of a small amount of non-glucose substance removable by yeast that no direct indication of the existence of this latter can be obtained from our data. The facts however are inconsistent with glucose being the sole fermentable or yeast-removable substance in urine. The second question is discussed later.

Recovery of added glucose from urine.

In view of the inconsistencies just reported we feel it necessary to give the actual recovery figures of small amounts of glucose from urine in which the clearing agent and the method of using the yeast are varied. Eagle [1927] had advised the use of yeast after H_2SO_4–Lloyd's reagent. Usually we have used yeast before the clearing agent. We determined added glucose when approximately 2·5 mg. glucose were added to a 2 hour fasting urine specimen and the "clearing" was varied as follows:

I. urine diluted and "cleared" by H_2SO_4–Lloyd's;
II. urine diluted and "cleared" by KH_2PO_4–MgO;
III. urine diluted and "cleared" by KH_2PO_4–MgO followed by H_2SO_4–Lloyd's reagent;
IV. urine diluted and "cleared" by $HgSO_4$–$BaCO_3$.

Table IV. *Showing recovery of small amounts of glucose added to normal fasting urine. The glucose is recovered as extra fermentable sugar.*

Figures represent mg./2 hours.

	Yeast used previous to "clearing"				Yeast used after "clearing"			
	Fermentable sugar present in urine Reagent		Glucose added 2·6 mg. Extra fermentable sugar Reagent		Fermentable sugar present in urine Reagent		Glucose added 2·7 mg. Extra fermentable sugar Reagent	
Method	1	2	1	2	1	2	1	2
I	4·4	3·0	2·8	3·1	3·2	4·2	2·9	2·6
II	3·6	2·6	3·4	2·8	5·5	3·8	2·8	2·8
III	3·8	3·8	2·9	2·8	3·4	4·9	3·4	2·2
IV	3·0	3·7	2·5	2·8	3·4	4·7	3·1	2·4

The preliminary dilution was approximately 1 : 3. The yeast was used on the preliminary diluted urine, before the clearing agent in one series of experiments and after the clearing agent in a second series. The final dilution was 1 : 6. The reducing power was determined by both copper reagents. All methods of clearing and variations in the use of yeast gave satisfactory recovery figures (Table IV).

Effect of dilution preliminary to clearing.

We have stated that to minimise in our previous experiments the effect of any possible non-glucose substances removable by yeast on our determinations of fermentable sugar in normal urine, we used a high dilution. Apart from the limits of our ability to estimate glucose, to what extent did the high preliminary dilution contribute to our previous negative findings?

A. A 2 hour specimen of normal fasting urine was diluted to 150 cc. (dilution approximately 1 : 3). On a portion of this were determined total and non-fermentable sugar, using H_2SO_4–Lloyd's reagent and allowing the yeast to act previous to clearing. The final dilution, at which the sugar determinations were made, was approximately 1 : 6. Except for a more concentrated urine, the technique follows exactly that of our previous paper.

A/3. Portions of the final 1 : 6 dilution urines from A were diluted with water to a dilution 1 : 18.

B. 50 cc. of the preliminary diluted (1 : 3) urine from A were diluted to 150 cc., making a new preliminary dilution of 1 : 9. On this were determined total sugar after H_2SO_4–Lloyd's reagent and non-fermentable sugar after yeast and H_2SO_4–Lloyd's reagent. The final dilution was 1 : 18. Copper reagents 1 and 2 were used in the three sets of determinations.

A comparison of the results from A, A/3 and B should show the same amount of fermentable sugar in mg./2 hours if glucose is the sole fermentable sugar in urine. Expressed in cc. 0·005 *N* thiosulphate per 2 cc. final dilution of urine, the fermentable sugar of both A/3 and B should be 1/3 the value of A, if we are dealing only with glucose. If, however, substances other than glucose constitute part of this fraction, the values for fermentable sugar in B might vary markedly from those of A/3.

The results are shown in Table V. The amount of fermentable sugar found in B is very much less than in either A or A/3. With reagent 1 the amount is less than one-half, in all but one sample. The use of reagent 2 shows 4 samples of B out of 6 to contain less fermentable sugar than A. The comparison of A and A/3 shows the latter in cc. 0·005 *N* thiosulphate to be almost exactly 1/3 the former.

Table V. *Showing the effect of the amount of dilution preliminary to the action of yeast and H_2SO_4–Lloyd's reagent on fermentable sugar in normal fasting urine.*

	Copper reagent 1			Copper reagent 2		
Subject	A	A/3	B	A	A/3	B
R.W.I.U.	0·31	0·10	0·09	0·32	0·12	0·07
C.E.D.	0·28	0·10	0·04	0·30	0·10	0·09
C.E.D.	0·33	0·12	0·02	0·27	0·09	0·06
R.A.	0·40	0·13	0·06	0·41	0·12	0·08
D.L.S.	0·25	0·08	0·07	0·23	0·08	0·05
G.A.G.	0·42	0·12	0·04	0·27	0·08	0·10
Average	0·33	0·11	0·05	0·30	0·10	0·07
mg./2 hours as glucose	7·0	7·0	3·2	5·2	5·2	3·6

Results in cc. 0·005 *N* thiosulphate per 2 cc. urine-filtrate.

The error of simple dilution is 0·01 cc. Again the facts are inconsistent with the unity of the fermentable sugar fraction of normal fasting urine.

Why do the results with reagent 1 in the present work fail to duplicate our previous results, and why does reagent 1 give lower, similar and higher results with reagent 2 in Tables III, IV and V? Whilst we have not entirely excluded the possibility that small unconscious differences in detail in the use of the copper solutions or the preparation of the filtrates may be responsible for the discrepancies, it seems more likely that inequalities in the preparation of reagent 1 are the cause. The figures for fermentable sugar in fasting urine as obtained by reagent 2 are relatively stable from preparation to preparation, and each preparation can be easily checked by reference to the p_H or titration values of the B solution (Harding and Downs). Reagent 1 has also been standardised as far as possible, and each preparation is always checked in value against pure glucose and found to give the same standard figure. This, however, is known to be no guarantee that each preparation will possess the same value to all other reducing substances. Harding and Van Nostrand [1930] noted variations in oxidising power to non-sugar reducing substances of whole blood. Harding and Selby drew attention to the same fact, and this instability of the preparation was one of the reasons that caused us to seek a different copper oxidising reagent with good general properties. The instability is brought about in the preparation of the reagent by the necessity of neutralising Na_2CO_3 by free tartaric acid, with a resultant uncertainty of the carbonate-bicarbonate ratio, though this can be controlled with sufficient accuracy to obtain constant results with glucose (see criticisms by Somogyi [1926]). The results for fermentable sugar in normal fasting urine and after glucose, both morning and afternoon, as reported by Harding and Selby in their previous paper, were all obtained with one large preparation of the copper reagent. Two or three preparations have been used in the work of the present paper. If our explanation is correct, a somewhat accidental circumstance combined with our high preliminary dilution was responsible for our previous negative findings for fermentable sugar in normal fasting urine. On the other hand, all the evidence of the present paper makes uncertain the amount of glucose present in such urines and favours the minimum rather than the maximum figures.

Effect of oral administration of glucose on urine "fermentable" sugar.

We have re-examined this question, using conditions favourable to showing a high sugar removal by yeast.

50 g. glucose in 400 cc. water were taken orally at 9 a.m. under the same conditions as in our previous paper. Some days later 50 g. glucose were taken by the same subjects at 4 p.m. (under conditions of afternoon glycosuria), the noon meal being of general mixed character. Blood-sugars were estimated at 9 a.m., 2 p.m. and 4 p.m., and every 15 minutes after the glucose administration. We have reported only the zero, peak and 2 hour values. All blood-sugars were determined on cutaneous blood [Herbert and Bourne, 1930]. Urine was cleared by KH_2PO_4–MgO followed by H_2SO_4–Lloyd's reagent, and also by $HgSO_4$–$BaCO_3$. Yeast was used after clearing and the reducing power determined by reagent 2. The final dilution was approximately 1 : 10.

Table VI shows that there is a small rise in urine "fermentable" sugar after oral glucose administration, whether given fasting at 9 a.m. or at 4 p.m. even when the peak of the blood-sugar does not rise above the normally accepted renal threshold. When the peak rises above the usual threshold value, as in cases C.E.D. and A.R.A., the usual large amounts of fermentable sugar enter the urine. Unless this latter occurs, the increases, however, are so small that

Table VI. *Showing effect of oral glucose on urine "fermentable" sugar.*

Subject	Morning or afternoon	Blood-sugar, mg./100 cc. 0 hour	Peak	2 hours	Urine "fermentable" sugar mg./2 hours Pre-glucose	Post-glucose
D.L.S.	M	77	124	97	5·0	6·4
					3·5	6·8
	A	94	144	81	11·0	11·4
					10·7	11·1
T.F.N.	M	87	130	85	3·8	4·5
					—	—
	A	107	173	94	9·2	14·6
					10·2	16·0
S.J.	M	80	132	66	2·7	7·0
					3·5	6·1
	A	93	149	109	4·6	7·1
					5·6	7·3
G.A.G.	M	90	134	82	5·8	7·0
					5·5	6·7
	A	86	173	87	9·1	13·2
					10·4	13·9
A.R.A.	M	107	150	60	4·7	6·1
					5·0	6·1
	A	92	232	123	2·4	317
					2·6	317
C.E.D.	M	97	154	91	4·1	5·2
					4·4	5·0
	A	105	204	105	5·7	338
					8·0	343

The first of each pair of urine "fermentable" sugar values is obtained after KH_2PO_4–MgO followed by H_2SO_4–Lloyd's reagent. The second of each pair is after $HgSO_4$–$BaCO_3$.

our previous conditions would have failed to detect them, unless we had adopted the extremely rigorous technique of Table I.

A correspondence between the blood-sugar level and the amount of urine "fermentable" sugar as determined after $HgSO_4$–$BaCO_3$ or KH_2PO_4–MgO and H_2SO_4–Lloyd's reagent is also shown in Table VII. The morning blood-sugar

Table VII. *Showing relation of fasting blood-sugar to level of urine "fermentable" sugar.*

Subject	Blood-sugar 9 a.m. mg./100 cc.	Urine-sugar 7–9 a.m. mg./2 hr.	Blood-sugar 2–4 p.m. mg./100 cc.	Urine-sugar 2–4 p.m. mg./2 hr.
D.L.S.	77	5·0*	115–94	11·0*
		3·5†		10·7†
S.J.	80	2·7*	88–93	4·6*
		3·5†		5·6†
T.F.N.	87	3·8*	122–107	9·2*
		—		10·2†
G.A.G.	90	5·8*	117–86	9·1*
		5·5†		10·4†
C.E.D.	97	4·1*	124–105	5·7*
		4·4†		8·0†
A.R.A.	107	4·7*	83–92	2·4*
		5·0†		2·6†

* Urine "cleared" by KH_2PO_4–MgO followed by H_2SO_4–Lloyd's reagent.
† Urine "cleared" by $HgSO_4$–$BaCO_3$.

at 9 a.m. is assumed as the level from 7–9 a.m. [Trimble and Maddock, 1929]. The blood-sugar level at 2 p.m. and 4 p.m. is usually higher than the fasting morning level. In all these cases the urine "fermentable" sugar is higher from 2–4 p.m. than from 7–9 a.m. The one exception where the 2–4 p.m. blood-sugar level is lower than in the morning shows a lower urine "fermentable" sugar.

The conclusion must be reached that fermentable sugar as obtained under such conditions is intimately connected with the level of the blood-sugar, or with the level of carbohydrate metabolism of which the fasting and post-prandial blood-sugar is also a reflex. In view of the facts set forth in the preceding sections showing the lack of unity of proof that glucose is the sole constituent of the fraction called urine "fermentable" sugar, we hesitate to conclude that the increases are glucose. The hesitation is strengthened if we tabulate the increases in urine "fermentable" sugar after glucose feeding in the order of the peaks of the blood-glucose. We should expect the two sets of figures to show some degree of correspondence if there is a simple escape of blood-glucose through the kidney. Table VIII shows such an arrangement. In making up the table

Table VIII. *Showing the peaks of blood-sugar and increase in urine "fermentable" sugar after glucose feeding.*

	Urine "fermentable" sugar		
Blood-sugar peak mg./100 cc.	Pre-glucose mg./2 hours	Post-glucose	Increase
124	4	6	2
130	4	4	0
132	3	6	3
134	6	7	1
144	11	11	0
149	5	7	2
150	5	6	1
154	4	5	1
173	9	13	4
173	9	15	6
204	7	340	333
232	2	317	315

we have taken the mean of the KH_2PO_4–MgO and the $HgSO_4$–$BaCO_3$ values as representing the urine "fermentable" sugar to the nearest mg. It will be seen that no close correspondence between increases in blood-sugar and urine "fermentable" sugar exists. The increases after glucose feeding, though distinct, are small and remain so until, in this small series, the peak of the blood-sugar reaches 170 mg. The increase at this point is much more marked than after the lower blood-sugar peaks. At a peak of 204 mg. or 232 mg. the urine becomes flooded with fermentable sugar. This is the familiar description of the renal threshold for glucose, with a slight variation. Our figures supply no evidence for a gradual progressive increase in urine "fermentable" sugar with increasing peaks in blood-glucose.

West, Lange and Peterson evidently intend it to be understood that urine "fermentable" sugar as determined by their method is dependent on the level of the blood-sugar. They draw attention to a sharp increase in fermentable sugar in a dog's urine occurring after meat feeding and remark, "it is probable also that the glucose of the blood reached a maximum about this time." Yet they ignore their findings that extremely irregular excretions of fermentable sugar apparently occur on two successive days of starvation in the same individual. Subject E.S.W. excretes 326 mg. fermentable sugar on normal diet, then 308 mg.

and 68 mg. on two starvation days. Subject H.L.W. puts out 38 mg. and 160 mg. fermentable sugar on two successive starvation days, the latter being slightly greater than after a day's normal diet. Such findings are inconsistent with a close correlation of blood-sugar and urine "fermentable" sugar. The blood-sugar during fasting exhibits no large fluctuations, such as are produced by the intake of food. Sugar remains at the normal fasting level with a slow fall as the fasting progresses from day to day.

Summary.

1. Ammonium salts, in physiological amounts, lower the total "sugar" and non-fermentable "sugar" found in urine, when the determinations are made by some of the sensitive Shaffer-Hartmann reagents. The fermentable sugar value may be unaltered.

2. The use of KH_2PO_4 followed by MgO on normal urines constitutes a method of clearing urine. The filtrates are suitable for "sugar" determinations.

3. KH_2PO_4–MgO can be used in conjunction with H_2SO_4–Lloyd's reagent for clearing urines previous to "sugar" determinations.

4. The values for fermentable sugar in normal fasting urine can vary with the clearing agent and with the copper reagent.

5. All the variations of method permit of the recovery of added glucose to urine, in the form of extra fermentable sugar.

6. The amount of fermentable sugar found in normal fasting urine can be varied by the state of dilution previous to the application of H_2SO_4–Lloyd's reagent.

7. Provided the method of clearing and the oxidising reagent correctly estimate small amounts of added glucose, the lower values for fermentable sugar should be accepted as more nearly correct.

8. Using the methods which give the higher results for fermentable sugar in urine, the oral ingestion of glucose results in slight but distinct increases in this fraction. These increases cannot be correlated with the increase in the blood-sugar. When the usual renal threshold for sugar is passed, large amounts of sugar appear in the urine.

9. We conclude that some substance removable by yeast other than glucose exists in normal human fasting urine.

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CCXIX. THE EFFECTS OF AMINES ON OXIDATIONS OF THE BRAIN.

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(*Received August 29th, 1933.*)

An investigation into the effects of narcotics on the oxidative processes of the brain has shown [Quastel and Wheatley, 1932, 1, 2] that these substances exert specific inhibitions at low concentrations. The oxidations of glucose, sodium lactate and sodium pyruvate are markedly affected whilst those of sodium succinate and *p*-phenylenediamine are unimpaired. This generalisation holds for all narcotics investigated, the inhibitory power varying, among narcotics of the same chemical type, with the hypnotic activity. A view of the mechanism of narcosis, which fits the observed facts, is that the narcotic is adsorbed from the blood-stream at a specific area or centre of the nervous system. Here it diminishes the ability of the cells constituting the nervous centre to activate lactic or pyruvic acid and in this way inhibits the oxidation by these cells of glucose, lactic or pyruvic acid. The activation and access of oxygen remain unimpaired, but, in consequence of the inhibition of oxidative powers, a lowered amount of energy is available to the narcotised nervous centre for the accomplishment of its functional activity. Narcosis—or a depression of the normal activity of the centre in question—may then ensue. In spite therefore of the fact that oxygen is freely available, a state equivalent to partial anoxaemia exists at those areas of the nervous centre where the narcotic is adsorbed. The results of experiments carried out under anaerobic conditions (oxygen being replaced by methylene blue) show that the adsorption of the narcotic is reversible, a competition taking place between lactic acid and the narcotic for possession of the active surfaces in the nervous centre [Davies and Quastel, 1932].

A development of this work now shows that there exist substances, normally produced in the body, whose effects on the oxidative processes of the brain closely resemble those of the narcotics. This result is of particular significance in the study of mental disorder. Recent investigations [McFarland, 1932] have indicated that the psychological reactions following anoxaemia and light narcosis closely resemble those found in certain psychotic disorders. It is not unreasonable to consider that if substances normally produced in the body have effects similar to those of the narcotics and can induce an anoxaemia in the nervous system, they may be held, when present in the blood-stream in more than ordinary quantities, partially responsible for psychotic manifestations.

The substances in question have been found to be chiefly degradation products derived from tyrosine and tryptophan.

Experimental method.

The technique employed in this investigation has been described fully in previous papers by the writers [1932, 1, 2] in which all details are available. The method consists, briefly, in allowing mixed (whole) brain tissue, usually of the guinea-pig, to take up oxygen at 37° in a Barcroft respirometer until the rate of uptake has fallen to between 50 and 75 % of the initial value. After this period (about 2½ hours) glucose, sodium lactate or other metabolite under investigation is added to the tissue and the new rate of oxygen uptake determined. This rate is compared with that obtained by brain tissue exposed from the commencement of the experiment to the substances whose narcotic effects are the subject of this study.

The experiments are carried out on 0·5 g. tissue (made as homogeneous as possible by chopping with a scalpel) in a saline-phosphate ($M/15$) medium at an initial p_H of 7·4. The amines *etc.* are dissolved in saline and brought to neutrality before adding to the brain tissue. Measurements of O_2 uptake are made at 15 minute intervals, all the Barcroft vessels containing air. Experiments have been carried out with brain (cortex) slices in an atmosphere of oxygen, but, since it appears a characteristic feature of brain that mincing or chopping in the manner described does not appreciably affect its oxidative mechanisms (so far as the substances mentioned in this paper are concerned) and since the magnitude of the Q_{O_2} of brain slices (in oxygen) is much the same as that of minced brain (in air), the writers have confined themselves almost exclusively to work with the minced tissue. The results which have been obtained with brain slices are in agreement with those with the minced tissue.

Results.

The amounts of oxygen consumed by 0·5 g. guinea-pig (whole) brain tissue, normally and when exposed to various amines (usually at a final concentration of 0·12 %), in presence of glucose, sodium lactate, pyruvate, succinate and

Table I. *mm.³ O_2 taken up in 2 hours at 37° by 0·5 g. guinea-pig brain tissue, normally, and in the presence of various amines, after 2½ hours' oxidation prior to the addition of glucose, sodium lactate* etc.

	Metabolite.					
Amine 0·12 %	Saline	Glucose 0·025 %	Sodium lactate 0·025 *M*	Sodium pyruvate 0·025 *M*	Sodium succinate 0·05 *M*	Sodium glutamate 0·05 *M*
None	287	615	721	785	1698	591
β-Phenylethylamine	303	462	594	520	1739	426
β-Phenyl-β-hydroxy-ethylamine	331	486	576	537	1808	482
β-3:4:5-Trimethoxy-phenylethylamine (Mescaline)	313	392	480	455	1744	436
Tyramine	242	285	296	293	540	275
Indole*	184	376	415	413	1558	221
*iso*Amylamine	208	287	302	342	1678	298
Neurine	290	672	848	788	1584	573
Cadaverine	317	570	686	613	1780	569
Putrescine	321	601	717	642	1660	540
Ethylamine	369	605	805	—	1585	—
Histamine†	315	670	813	877	1700	652

* 0·5 cc. of a saturated solution of indole in saline at 37° was added to the brain tissue in the saline-phosphate medium, the final volume being 4 cc.

† Present as 0·12 % histamine acid phosphate (neutralised).

glutamate are recorded in Table I. The amounts given are those found in typical experiments and afford a basis for comparison of the inhibitory effects of certain amines on brain oxidations. They are the values obtained after the brain tissue had been allowed largely to deplete itself of oxidisable material by shaking in air at 37° for 2½ hours prior to addition of the metabolites under investigation. The amines had been added to the brain at the commencement of the experiment so that exposure to these substances had occurred for 2½ hours before metabolites were added.

Normally under these experimental conditions constant and reproducible results may be obtained [Quastel and Wheatley, 1932, 1]. The values of the amounts of oxygen taken up by untreated guinea-pig brain in presence of various metabolites are shown on the first line of Table I. The rest of this table shows the effects of exposure of the brain tissue to various amines on the amounts of oxygen consumed. Calculations of the percentage inhibitions effected by these amines on the extra oxygen uptakes due to the addition of metabolites are shown in Table II. Included in Table II, for purposes of comparison, are

Table II. *Percentage inhibition by amines of extra oxygen uptakes due to various metabolites.*

Amine 0·12 %	Glucose	Sodium lactate	Sodium pyruvate	Sodium succinate	Sodium glutamate
β-Phenylethylamine	52	33	44	0	60
β-Phenyl-β-hydroxyethylamine	53	44	54	0	50
Mescaline	76	62	72	0	59
Tyramine	87	88	88	79	89
Indole*	41	47	54	3	87
*iso*Amylamine	76	79	73	0	70
Neurine	0	0	0	8	7
Cadaverine	26	15	41	0	17
Putrescine	15	9	36	5	28
Histamine	0	0	0	2	0
Narcotic 0·12 %					
Allyl*iso*propylbarbituric acid	73	71	67	2	28
Phenylethylbarbituric acid	94	79	85	0	50
Hyoscine	79	73	71	0	60
Chloral	66	90	90	0	62

* See footnote, p. 1610.

the percentage inhibitions of oxygen uptakes effected by typical narcotics—allyl*iso*propyl- and phenylethyl-barbituric acids, hyoscine and chloral—the experiments being carried out in the same manner as for the amines.

A characteristic feature of narcotic activity is the large inhibitory effect on the oxidations of glucose, sodium lactate and pyruvate, and little or none, at the concentrations used, on that of sodium succinate. It will be observed from Tables I and II that there exists a similar behaviour on the part of a number of amines.

Whilst cadaverine, putrescine, neurine and ethylamine have relatively little action on the oxidation of glucose or sodium lactate at the concentrations used, tyramine, β-phenylethylamine and β-phenyl-β-hydroxyethylamine have relatively large inhibitory effects. Mescaline (β-3 : 4 : 5-trimethoxyphenylethylamine), well known for its production of visual hallucinations and used of late in psychiatric studies, has similar large effects. Indole[1] and *iso*amylamine also have considerable inhibitory actions, but histamine is without effect.

[1] Skatole is also highly effective in diminishing the oxidation of glucose by brain.

A notable feature is the exceptionally toxic action of tyramine. Whilst the other derivatives of tyrosine exert specific effects similar to the narcotics, tyramine has a considerable inhibitory action on the oxidation of sodium succinate. This would indicate a more widespread inhibition of the oxidative changes of the brain by tyramine than by the basic amines and narcotics so far investigated. Another point of some interest is the inhibitory action of cadaverine and putrescine on the oxidation of sodium pyruvate with relatively little effect on that of glucose and sodium lactate.

The results as a whole show clearly that amines of the aromatic type, together with *iso*amylamine, exert effects upon brain oxidations which resemble those of the narcotics. There is the same relatively large inhibitory action[1] on the oxidation of glucose and sodium lactate and, except in the case of tyramine, a lack of effect on the oxidation of sodium succinate. The oxidation of sodium glutamate is also considerably affected by the amines and by narcotics. The magnitude of the effects is of the same order as that of the narcotics at the concentrations used.

Whilst the explanation of this behaviour of the amines is probably similar to that of the narcotics, *i.e.* a competition with lactic acid *etc.* for the active surfaces involved, the practical result is of some importance.

Many of the substances studied are normally produced in the bacterial breakdown of amino-acids. Their passage into the liver after absorption into the blood from the intestine is followed by their detoxication—either conjugation as in the case of indole or oxidation as in the case of tyramine. A disturbance in the hepatic detoxicating mechanisms might lead to the circulation in the blood of more than the ordinary quantities of these bases, and this would be expected to lead ultimately to the production of psychological reactions similar to those met in anoxaemia or in the early stages of narcosis. It is not unreasonable therefore to suspect a disturbance in hepatic function as a causative element in certain types of mental disorder. Investigations of the metabolic events in the liver and their possible variations in certain psychotic conditions are clearly necessary and such work is now in progress.

Summary.

1. An investigation has been made of the effects of a number of amines on the oxidations of brain tissue.

2. β-Phenylethylamine, β-phenyl-β-hydroxyethylamine, tyramine, indole, *iso*amylamine and mescaline inhibit strongly the oxidation of glucose, sodium lactate and sodium pyruvate by the brain. They also inhibit the oxidation of sodium glutamate but, with the exception of tyramine, they have little or no effect on the oxidation of sodium succinate. Tyramine presents the only instance so far of an amine or narcotic affecting succinate oxidation at the concentration used. The effects of these amines are similar to, and are of the same order of magnitude as, those of typical narcotics (*e.g.* luminal, hyoscine, chloral).

3. Neurine, cadaverine, putrescine, ethylamine and histamine have relatively little effect on the oxidation by the brain of glucose, sodium lactate or sodium succinate.

[1] The inhibition decreases with decrease in concentration of amine (or of narcotic), definite inhibitions being observed at concentrations considerably lower than 0·12 %. The latter concentration was chosen to give results quite outside experimental error and clearly to indicate the specificity of behaviour.

4. The bearing of these results on the study of certain types of mental disorder is considered.

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CCXX. STUDIES IN ZYMASIS[1].

IV. THE ACCUMULATION OF ZYMASIC PRODUCTS IN APPLES DURING SENESCENCE.

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Zymasis may be measured either in terms of the CO_2 which escapes or in terms of the products which accumulate[2]. In the work described in the present and in the next two papers we have usually employed the "alcohol number" to evaluate zymasis. This number denotes the sum of the concentrations (in g. per unit mass fresh weight of tissue) of ethyl alcohol and of acetaldehyde found in the fruit by analysis at any time.

The apples used throughout this work were stored for us in air for two seasons (1929–30 and 1930–31) at the Low Temperature Research Station, Cambridge, and for one season (1931–32) at the Ditton Laboratory, East Malling, whence they were sent to Newcastle by post[3].

In the present paper we report the changes we have found in the alcohol number by analysing apples (*a*) immediately after their arrival from low temperature stores at different times during the storage season (see Table I and Fig. 1), and (*b*) after subsequent exposure to air under the experimental conditions employed in the work described in the next two papers[4] (see Table II and Fig. 2).

This is the first time that data concerning zymasis in air by apples have been collected throughout a storage season.

It had earlier been reported that whereas zymasic products do not accumulate in Newton Wonder apples in early storage [Thomas, 1925], zymasis occurs in several varieties of apples and pears during the oxidase browning that follows injury or physiological disease [Thomas, 1929]. So far as we know, however, no data have been published for older apples before browning begins, although for Bartlett pears it is known that "ripening" is accompanied by a type of zymasis during which acetaldehyde accumulates in relatively high concentrations [Harley and Fisher, 1927].

[1] The following three papers constitute Parts I, II and III of "Studies in Zymasis": Thomas, *Biochem. J.* 1925, **19**, 927; *Ann. Appl. Biol.* 1929, **16**, 444; 1931, **18**, 60.

[2] By zymasis we mean the process of zymase cleavage of carbohydrates that leads to the production and accumulation of ethyl alcohol and often of acetaldehyde (but in relatively smaller amounts) in plant tissues and the simultaneous production and escape of carbon dioxide.

[3] We are indebted to Dr Kidd of Cambridge, and Dr West of East Malling, for arranging that these apples should be stored for us in rooms kept at a constant temperature at these Stations.

[4] The measurements of the alcohol number which are given in Section III constitute the control figures to the experiments carried out during the storage seasons 1929–30, 1930–31, 1931–32 on the production of ethyl alcohol and acetaldehyde by apples in nitrogen, and in nitrogen-oxygen mixtures. The results of these experiments form the subject matter of papers V and VI of this series.

I. Material.

Newton Wonder and Bramley's Seedling apples picked from trees in Mr Caleb Lee's orchard at Swanley, Kent, have been used in the major experiments which are described in this and in the two following papers. The apples were stored for us in air at 1° in the season 1929–30, and at 4° in the seasons 1930–31 and 1931–32. Samples have been sent to us by post for these researches[1].

II. Methods.

(i) *The estimation of the alcohol number.*

The method of estimation of the alcohol number will be reported in a later paper in this series. It is essentially that used by Thomas [1925], but modified so that ethyl alcohol and acetaldehyde may be estimated in the same steam distillate.

In the present paper we generally express our results in terms of the "percentage alcohol number." This means the sum in g. of the masses of ethyl alcohol and of the ethyl alcohol equivalent of acetaldehyde found in 100 g. fresh weight of fruit at the time of the analysis. The data in Table III, show that in these experiments the amount of acetaldehyde which accumulated was usually negligible when compared with that of ethyl alcohol. Hence, in the present work, the alcohol number is virtually a measure of the ethyl alcohol content of the fruit[2].

(ii) *Experimental procedure.*

When apples were received from cool storage, a sample was immediately analysed to determine the percentage alcohol number, *i.e.* the amount of zymasic products which had accumulated in 100 g. fresh weight of the fruit during storage at 1° or 3–4°, *plus* a day in the post. This number will be termed the percentage alcohol number of the "storage controls."

The remaining apples were then used to determine the percentage alcohol numbers of what we shall term in this present paper and in Part VI of this series the "conditions controls." To obtain this number, the apples, before they were analysed, were exposed for a period to the conditions stated below.

The sample of fruit was weighed and placed under a glass hemisphere with a wide equatorial flange, which was then fixed to a ground glass plate by means of paraffin wax. Through the pole of the container passed inlet and outlet tubes for the air stream. The fruit chamber was secured to an iron frame and immersed in a water-bath, the temperature of which was kept within 0·2° of 23° by means of a thermostat. CO_2-free air was drawn through the chamber at the rate of 2 litres per hour for a period of 120 to 140 hours. The issuing gas was bubbled through 50 cc. of concentrated sulphuric acid in a wash-bottle to trap any transpired alcohol. At the end of the period of exposure to the higher temperature, the fruit was re-weighed and analysed. The acid from the wash-bottle was added to aqueous potassium dichromate, and when oxidation was complete the mixture was distilled. The amount of alcohol transpired was calculated from the acetic acid value of this distillate. In Table II allowances have been made for this transpired alcohol.

[1] We are deeply indebted to Dr West for arranging to secure these varieties from a single orchard, and for sending samples from the air stores at the times we wanted them for our experiments.

[2] This is not always the case. Thus, for example, after CO_2-zymasis [Thomas, 1925], the ratio ethyl alcohol/acetaldehyde is sometimes less than 2/1. The alcohol number would then be over 50 % greater than the ethyl alcohol content of the fruit.

III. Results.

The results for the storage controls are given in Table I and Fig. 1. Table II and Fig. 2 deal similarly with the conditions controls. In Table III are given the amounts of acetaldehyde and ethyl alcohol found in apples at different times during the season 1931–32. The figures in this table have been extracted from the figures for the percentage alcohol number given in Tables I and II.

Table I (see Fig. 1). *Percentage concentration of products of zymasis in air-stored apples (storage controls).*

Variety of fruit	Date	Days in store	Percentage alcohol number	Remarks (state of fruit, *etc.*)
Newton Wonder:				
Season 1929–30	21. iv. 30	195	0·008	Sound
	16. v. 30	225		
	5. vi. 30	250	0·030	Superficial scald
	12. vi. 30	255	0·025	,,
	19. vi. 30	265	0·030	,,
	25. vi. 30	270	0·025	,,
Season 1930–31	17. vii. 30	From orchard	0·004	Very young. Green. Av. wt. 12 g.
	25. ix. 30	—	0·003	Young. Green. Av. wt. 120 g.
	20. x. 30	27	0·005	Green-yellow. Sound
	9. i. 31	105	0·010	Yellow-green. Wilted
	15. i. 31	112	0·011	,,
	23. i. 31	120	0·020	,,
Season 1931–32	1. vii. 31	From orchard	0·004	Very young. Green. Av. wt. 8·5 g.
	18. vii. 31	,,	0·004	,, 20·0 g.
	30. viii. 31	,,	0·004	,, 70·0 g.
	28. x. 31	28	0·005	Hard. Green
	9. xii. 31	70	0·003	Green to yellow-green
	6. i. 32	97	0·007	Yellow. Internal breakdown
	27. i. 32	119	0·019	Green-yellow. First sign of wrinkling
	25. ii. 32	147	0·018	Yellow. Wilted
Bramley's Seedling:				
Season 1930–31	13. xi. 30	50	0·015	Hard. Green
	20. xi. 30	55	0·010	,,
	7. i. 31	105	0·015	Green
	21. i. 31	120	0·030	Green. Wilted. Keeping badly, soft spots on fruit
	28. i. 31	127	0·033	Green-yellow. Slightly wilted
	6. ii. 31	135	0·025	,,
	11. ii. 31	142	0·030	Green-yellow. Badly wilted
	4. iii. 31	165	0·050	Badly wilted and scalded
Season 1931–32	1. vii. 31	From orchard	0·003	Small. Green. Av. wt. 23 g.
	18. vii. 31	,,	0·004	,, 40 g.
	30. viii. 31	,,	0·004	Young. Green. Av. wt. 125 g.
	10. xi. 31	40	0·018	Green
	6. i. 32	98	0·014	,,
	16. iii. 32	170	0·025	Green-yellow
	1. iv. 32	185	0·045	Green-yellow. First sign of wilting

IV. Conclusions.

(i) *The increase in ageing apples of the alcohol number of storage controls.* The data reported in Table I, and charted in Fig. 1 confirm the earlier observations of Thomas [1925] that the concentration of zymasic products in Newton Wonder apples early in the storage period does not rise above 0·005 % in cool storage at 1–4°. The alcohol numbers for the storage controls of Bramley's Seedling apples during this same period were usually higher than those for

Table II (see Fig. 2). *Increase in the percentage alcohol number in apples in air at 23° in the respiration chamber (conditions controls).*

No. of exp.	State of fruit	Date exp. begins	Duration of exp. (hrs.)	No. of apples in sample	Wt. of sample		Percentage alcohol number after 100 hrs.' exposure to air at 23° following low temperature storage (allowing for transpired alcohol)	Increase in percentage alcohol number during 100 hrs.' exposure to air at 23°
					Initial g.	Final g.		
1	2	3	4	5	6	7	8	9
Newton Wonder.								
Season 1930–31:								
7	Wilted. Superficial scald	11. vi. 30	114½	1	—	72·0	0·150	0·120
Season 1930–31:								
20	Green. Hard. Young	1. x. 30	142	1	—	100·5	0·017	0·014
A	Yellow-green, wilted	9. i. 31	117½	1	—	133·5	0·025	0·015
B	Yellow, wilted	16. i. 31	121½	1	—	97·5	0·150	0·140
Season 1931–32:								
76	Green. Hard. Very young	8. vii. 31	116¼	12	67·0	60·0	0·024	0·02
C	,,	17. vii. 31	113	6	134·5	132·5	0·019	0·014
84	,,	26. viii. 31	122	3	212·0	201·0	0·019	0·015
98	Green	28. x. 31	140	2	321·0	312·5	0·011	0·007
112	Green to yellow-green	10. xii. 31	141½	2	279·0	270·0	0·013	0·010
128	Green-yellow; first sign of wrinkling	27. i. 32	140½	1	97·5	97·0	0·034	0·015
H	,,	10. ii. 32	119½	1	—	—	0·077	0·060
133	,,	24. ii. 32	138	1	87·0	83·5	0·040	0·022
Bramley's Seedling.								
Season 1930–31:								
D	Green. Hard	20. xi. 30	118½	1	141·0	138·0	0·022	0·013
E	Yellow-green, slightly wilted	23. i. 31	117	1	—	125·5	0·090	0·060
46	,,	29. i. 31	116	1	—	170·0	0·093	0·060
47	,,	29. i. 31	116	1	—	130·0	0·100	0·060
48	Green-yellow	30. i. 31	116½	1	—	177·0	0·076	0·043
49	Yellow-green	30. i. 31	116½	1	—	160·0	0·073	0·037
55	Yellow. Badly scalded	5. iii. 31	116	1	—	147·0	0·200	0·150
56	,,	6. iii. 31	118	1	—	233·0	0·220	0·170
Season 1931–32:								
75	Green. Hard. Very young	8. vii. 31	116¼	8	172·0	161·5	0·010	0·007
F	,,	17. vii. 31	113	5	192·5	189·5	0·010	0·007
88	,,	2. ix. 31	140½	2	218·5	218·0	0·016	0·012
105	Green	11. xi. 31	115¾	1	207·5	202·0	0·070	0·052
120	,,	6. i. 32	138½	1	158·0	157·5	0·074	0·060
137	Green-yellow	16. iii. 32	140	1	167·5	164·0	0·110	0·090
141	Green-yellow; first sign of wilting	31. iii. 32	116½	1	127·5	126·0	0·100	0·050

Up to May 1931, the weight used in arriving at the percentage concentration of products of zymasis was the final weight of the apple. After this it was realised that the use of the final weight was open to objection, and since then the initial weight has been used. The loss of weight is never more than 6 % in a sound apple and is usually of the order of 1 to 3 % and, as all the figures in any series are calculated to the same basis, the error is not serious.

The original experimental numbers are retained for the convenience of such readers as may have access to Fidler's Ph.D. thesis [1932].

The percentage alcohol numbers given in column 9 represent the difference between column 8 and the corresponding percentage alcohol numbers in Table I. They constitute the conditions control values which were used as a basis for measuring zymasis owing to oxygen shortage in the work which is described later [Thomas and Fidler, 1933, Section II].

Newton Wonder apples, but did not significantly exceed 0·015 %. We infer that the rate of production of ethyl alcohol in storage controls during the earlier phase of storage is very slow. If zymasis and cell oxidations are linked processes, this slow rate may be due to the vigour with which some precursor of ethyl alcohol is oxidised during this phase.

Table III. *Production of acetaldehyde and ethyl alcohol in air by Newton Wonder and Bramley's Seedling apples during 1931–32.*

Time of analysis	Variety of apple	Number of exp.	Date	g. per 100 g. apple: Acetaldehyde	g. per 100 g. apple: Ethyl alcohol
When received from store	Newton Wonder	—	1. vii. 31	0·0005	0·003
		—	18. vii. 31	0·0006	0·003
		—	30. viii. 31	0·0005	0·004
		—	28. x. 31	0·0003	0·004
		—	9. xii. 31	0·0004	0·003
		—	6. i. 32	0·0005	0·007
		—	27. i. 32	0·0008	0·018
		—	25. ii. 32	0·0028	0·015
	Bramley's Seedling	—	1. vii. 31	0·0002	0·003
		—	18. vii. 31	0·0005	0·003
		—	30. viii. 31	0·0010	0·003
		—	10. xi. 31	0·0009	0·017
		—	6. i. 32	Nil	0·014
		—	16. iii. 32	0·0019	0·024
		—	1. iv. 32	0·0026	0·042
Following 100 hours' exposure to air at 23°	Newton Wonder	76	8. vii. 31	0·0018	0·022
		84	26. viii. 31	Nil	0·019
		98	28. x. 31	0·0007	0·011
		112	10. xii. 31	0·0006	0·013
		128	27. i. 32	0·0036	0·030
		H	10. ii. 32	0·0032	0·074
	Bramley's Seedling	75	8. vii. 31	0·0005	0·009
		88	2. ix. 31	0·0006	0·015
		105	11. xi. 31	0·0024	0·067
		120	6. i. 32	0·0033	0·071
		137	16. iii. 32	0·0043	0·108
		141	31. iii. 32	0·0047	0·090

The change in colour of an apple to a yellow-green, and, at a later date, the incidence of severe wilting and superficial scald, which occurs when the apple is full yellow, are the only external indications of the physiological state of the fruit. In wilted apples the skin can readily be pushed into ridges and later becomes noticeably wrinkled. Superficial scald means that there are dry brown patches just under the skin: this condition may be succeeded by deep scald, in which the whole of the skin is involved, and the browning extends deeply into the flesh of the fruit.

It will be seen from Fig. 1 that as Newton Wonder and Bramley's Seedling apples change in colour from green to green-yellow, the alcohol numbers of the apples rise, and as the apples become more yellow and wilted, the graph of the alcohol number takes on a steep upward path[1]. The data of Table III indicate that this rise in the alcohol number is compounded of a rise in both the ethyl alcohol and acetaldehyde contents of the apples. The highest recorded values for the percentage alcohol numbers in apples which show no browning, from an air store, are 0·03 in Newton Wonder apples in June 1930, and 0·05 in Bramley's Seedling apples in March 1931.

[1] A few experiments permit us to compare the Cleopatra apple, a variety shipped from overseas, with these two varieties of home-grown apples. Thus whereas the percentage alcohol number of green-yellow Cleopatras was less than 0·007, this number had increased to 0·023 by the time this variety had turned yellow. We note that the alcohol number of apples showing no browning from ships' holds which have been well ventilated may indicate the occurrence of recent zymasis. But there is no evidence yet of the alcohol number in apples from well ventilated air stores reaching the magnitudes found in the flesh tissue after faulty ventilation [Thomas, 1931].

It appears, therefore, that the rate of zymasis after a slow initial period of varying extent becomes increasingly accelerated as the apples age further. Possibly there is always a critical phase in the storage life of the fruit when certain changes become autocatalytic. Thus in a rough grouping of Newton Wonder apples we might place in one class those apples in which the percentage alcohol number is not greater than 0·005, and in the other those apples in which the alcohol number is steadily increasing beyond this figure. Similarly, for Bramley's Seedling apples, the line of demarcation may be placed at 0·015. In our view apples in the second class (*i.e.* those which have reached the critical phase) cannot be regarded as physiologically healthy, for, in our experience, when zymasis becomes relatively rapid the browning of the flesh tissue is not far distant.

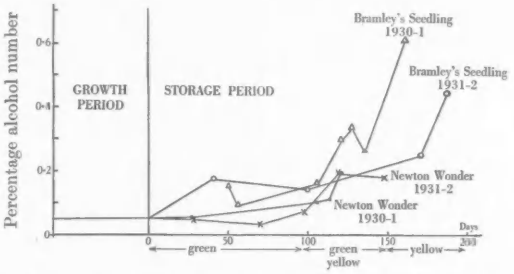

Fig. 1. Increase in percentage alcohol number in apples during storage.

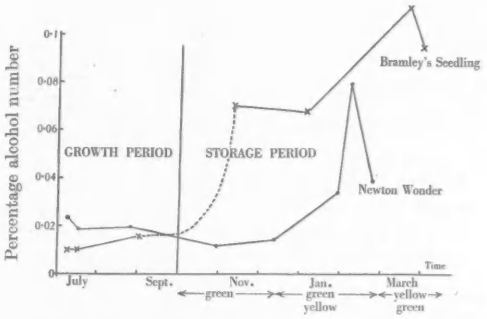

Fig. 2. Percentage alcohol number in apples after 100 hours' exposure to air at 23°, following (*a*) removal from the tree during the growing period, or (*b*) low temperature storage (1930–31)[1].

(ii) *The increase in ageing apples of the alcohol number of conditions controls.* The data in column 9 in Table II and the differences in the heights above the x-axis of comparable points in Figs. 1 and 2, show that the effect of placing

[1] We have never observed the alcohol number of an apple to rise until some months after it is removed from the tree. Accordingly, part of the graph for Bramley's Seedling apples during 1931–32 has been drawn with a broken line at a level which we consider approximately to represent the true state of affairs.

Newton Wonder and Bramley's Seedling apples[1] in a closed space in moving air at 23° at any time in the storage season is to increase the alcohol number[2]. Thus, for example, except in young Bramley's Seedling apples, the alcohol number for apples after 5 days' exposure to air, under experimental conditions at 23°, is higher than in apples received from a 4° store 1 to 2 months later. The cause of this may only be suggested. The two factors that, either together or singly, may influence zymasis are humidity and temperature. Of the effect of humidity as a single factor, nothing is known. It may well be that the question is related to temperature alone. Although the temperature of the experiment is 20° above that of the store, yet it is only 8° above the average temperature of the laboratory, so that the oxygen content of the intercellular spaces of the fruit should still be adequate for aerobic respiration.

It is well known that increase in temperature accelerates the rate of senescence in apples. Possibly one of the features of senescence is the progressive retardation of oxidative processes, and this may be the factor which leads to the observed increase in the alcohol number.

Changes in the alcohol number of the conditions controls at different periods in the storage season point to the same conclusions that we reached from the data given in the last sub-section. Thus the form of the graphs for the conditions controls is similar to that of the graphs in Fig. 1 for the storage controls, the rise in the alcohol number occurring at the same state of the fruit[3]. Once more the results (see Table II) may be divided into two groups. Before a certain critical phase the alcohol number of a conditions control, although higher than that of a storage control, remains relatively low. Later, when senescent characters are seen the disturbance in metabolism leading to zymasis becomes increasingly marked, as is evidenced by the rapid increase in the alcohol number of the conditions controls.

Summary.

1. Little zymasis occurs in Newton Wonder and Bramley's Seedling apples at the beginning of the storage season and up to the beginning of the change from green to yellow-green, when these varieties are stored in air at 1–4°.

2. Senescence is accompanied by a change in the respiratory metabolism of the cells of the fruit, leading to the accumulation in an apple stored in air of considerable amounts of ethyl alcohol and smaller amounts of acetaldehyde, *i.e.* the alcohol number of the apple rises.

3. Increase of temperature, which is known to accelerate the rate of senescence in an apple, leads to an increase in the alcohol number of the apple.

The work described in this, and in the following paper, was carried out in the Botany Department, Armstrong College, under the supervision of

[1] The Cleopatra apple responded similarly to this transfer to experimental conditions, thus in green to green-yellow apples the percentage alcohol number increased by 0·005 during 100 hours' exposure to air under experimental conditions at 23°.

[2] Hence, to eliminate the effect of experimental conditions as such, conditions controls and not storage controls should always be used in quantitative researches in which zymasis is induced by shortage of oxygen or by the presence of foreign substances [see *e.g.* Thomas and Fidler, 1933].

[3] It is not claimed that zymasis in air at 23° may be calculated from Fig. 2. Regarding the increased zymasis in air, there are two alternatives: (*a*) that the increased rate may be maintained over long periods, and (*b*) that the increase may take place only at the beginning of the exposure to the new experimental conditions. The present research is not concerned with the elucidation of these questions.

Mr Meirion Thomas, to whom I am indebted for continued advice and helpful criticism. My thanks are due to Armstrong College for a College Research Studentship during 1929–30, and to the Department of Scientific and Industrial Research for maintenance grants during 1929–30, and 1930–31, and for payment as a Research Assistant during 1931–32.

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CCXXI. STUDIES IN ZYMASIS.

V. SEASONAL FLUCTUATIONS IN ZYMASIS AND IN CARBON DIOXIDE/ALCOHOL NUMBER RATIOS IN APPLES IN THE ABSENCE OF OXYGEN.

By JOHN CARTER FIDLER.

From the Department of Botany, Armstrong College, Newcastle-upon-Tyne.

(*Received July 26th, 1933.*)

Towards the end of the nineteenth century it was realised that the cells of many higher plants could form ethyl alcohol in the absence of oxygen [see literature quoted by Kostytschew, 1927], and several workers made simultaneous measurements of the production of ethyl alcohol and CO_2 in anaerobiosis.

Recent comparisons of anaerobic and aerobic metabolism have led to an alteration of biological outlook on cell respiration in animals, yeast and bacteria. It was shown by Blackman [1928] that the cells of higher plants fall into line with those of other organisms. Blackman based his conclusions on measurements of the CO_2 output of stored Bramley's Seedling apples in air and in nitrogen. At that time he made the primary assumption in his paper that the ratio CO_2/ethyl alcohol in the anaerobic respiration of this variety is unity. For example, he wrote, "in nitrogen, Group D (by which he implies intermediate compounds between hexoses and ethyl alcohol) proceed quantitatively to the two final products CO_2 and alcohol in the usual ratio," and, "it will be held that for one atom of carbon thus detected (as CO_2) there are two atoms of carbon excreted into the tissues as alcohol." Actually, as will be pointed out later, the precise value of the ratio CO_2/ethyl alcohol, although affecting his arithmetical deductions slightly, does not influence his main argument. Some of his conclusions, however, are only valid provided the ratio, whatever may be its magnitude, keeps constant throughout the storage season. It was the aim of the present investigation to test whether this was the case. The quantitative methods of estimating ethyl alcohol and acetaldehyde, which will be described in a later paper, have been employed to determine the CO_2/alcohol number [see Fidler, 1933] ratio in selected varieties of apples placed under anaerobic conditions at different times during the storage season. The results for Bramley's Seedling and Newton Wonder apples are given in Section I, and the results of preliminary measurements of the CO_2/alcohol number ratio in several other varieties of apples are shown in an appendix.

Materials and methods.

The apples used were from the population used throughout this work [Fidler, 1933]. They were placed in the apparatus already described [Fidler, 1933] at a temperature of 23°. The gas stream, after passing through the fruit chamber, bubbled through a wash-bottle containing sulphuric acid (as described in the first paper), and then through a Pettenkofer tube containing 60 cc. of standard baryta. These tubes were changed at intervals.

After the apples had attained the temperature of the bath, air was drawn through at a rapid rate for a period varying between 18 and 24 hours. The air-line thus being fixed, the air current was changed for pure nitrogen at the standard rate of 2 litres per hour[1].

The nitrogen used in the experiments was commercial cylinder nitrogen, and was found to contain between 0·5 and 1·2 % oxygen. This was removed by passing the gas over copper which was heated electrically to 650–700°. Gas analyses of the purified gas showed that this treatment reduced the percentage of oxygen to less than 0·05.

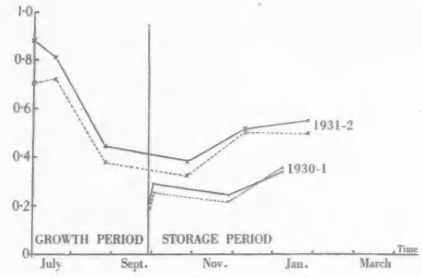

Fig. 1. Rates of CO_2-production (——), and rates of increase in the alcohol number (· · · ·) in Newton Wonder apples in nitrogen at 23°. Ordinate: g. CO_2 produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours.

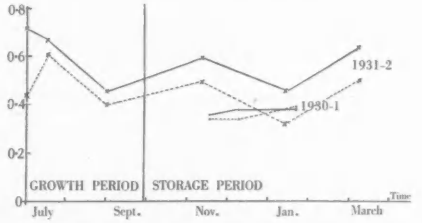

Fig. 2. Rates of CO_2-production (——), and rates of increase in the alcohol number (· · · ·) in Bramley's Seedling apples in nitrogen at 23°. Ordinate: g. CO_2 produced by 100 g. fruit during 100 hours or increase in alcohol number during 100 hours.

At the end of the exposure to the gas (4 to 5 days), the fruit was removed from the apparatus, weighed, and the alcohol number was estimated. The CO_2 absorbed by the baryta was estimated by titrating the barium hydroxide-carbonate mixture against standard hydrochloric acid using phenolphthalein as an indicator.

Part of the same sample of fruit was analysed immediately on being received, and the alcohol number thus obtained was subtracted from that found in the above experiment, in order to get a measure of the effect of the absence of oxygen on zymasis.

[1] The rate of passage of the gas stream was measured in the first instance by collecting the emergent gas in a graduated jar over water. The rate of bubbling through a wash-bottle containing KOH (to remove CO_2 from gas entering the apparatus) was then noted. This rate of bubbling was reproduced in subsequent experiments.

Table I. *CO_2/alcohol number in apples in pure nitrogen at 23°.*
(See Figs. 1 and 2.)

	No. of exp.	Variety of fruit	State of fruit	Date exp. begins	Duration of exposure to nitrogen (hrs.)	No. of apples in sample	Wt. of sample (g.) Initial	Wt. of sample (g.) Final	Percentage CO_2 production (g./100 hrs.)	Increase in percentage alcohol number during 100 hrs.' exposure to nitrogen, allowing for transpired alcohol	Ratio CO_2/alcohol number (both expressed as gram-molecular equivalents)*	Glycolysis in C units/CO_2 in C units†
	1	2	3	4	5	6	7	8	9	10	11	12
Season 1929–30:												
Not plotted	1	Newton	Sound	1. iv. 30	121½	1	—	99·5	0·412	0·37	100 : 86	2·7
Not plotted	3	Wonder	Superficial scald	14. v. 30	123¾	1	—	88·5	0·426	0·40	100 : 91	2·8
Season 1930–31:												
	17	Newton	Green. Hard	24. ix. 30	97½	1	—	112·5	0·203	0·19	100 : 89	2·8
	21	Wonder	Young. Hard	1. x. 30	100¼	1	—	144·0	0·290	0·25	100 : 84	2·7
	30		Green to green-yellow	27. xi. 30	97	1	—	193·0	0·242	0·21	100 : 84	2·7
	37		Yellow, slightly wilted	8. i. 31	99½	1	—	105·0	0·338	0·35	100 : 99	3·0
Season 1931–32:												
	73	Newton	Green. Hard. Very young	1. vii. 31	97¾	12	103·0	96·5	0·887	0·70	100 : 75	2·5
	79	Wonder	,,	17. vii. 31	95	9	174·5	167·0	0·813	0·73	100 : 86	2·7
	81		,,	26. viii. 31	98½	3	208·0	201·0	0·448	0·38	100 : 81	2·6
	95		Green	28. x. 31	99	2	252·5	246·0	0·384	0·32	100 : 80	2·6
	109		Green to yellow-green	10. xii. 31	98½	2	298·5	293·5	0·520	0·50	100 : 93	2·8
	125		Green-yellow. First sign of wilting	27. i. 32	99	1	84·0	81·5	0·549	0·50	100 : 88	2·7

Season 1932–33:											
	143	Newton Wonder	Small and young	16. viii. 32	120	4	276·0	273·5	0·264	0·19	100 : 69	2·4
Not plotted I	*a*	Newton Wonder	Green	19. x. 32	119	2	260·0	254·5	0·254	0·15	100 : 56	2·1
	b		,,	19. x. 32	119	2	306·0	301·5	0·276	0·18	100 : 60	2·2
	c		,,	19. x. 32	119	2	296·0	291·5	0·316	0·20	100 : 66	2·4
III	*a*		,,	10. xi. 32	95½	2	226·5	225·5	0·343	0·24	100 : 68	2·4
	b		,,	10. xi. 32	95½	2	226·0	222·0	0·315	0·24	100 : 74	2·5
	c		,,	10. xi. 32	95½	2	235·0	230·5	0·329	0·24	100 : 70	2·4
Season 1930–31:												
	25	Bramley's Seedling	Green. Hard. Young	19. xi. 30	97¾	1	—	148·5	0·356	0·34	100 : 91	2·8
	34		Green. Firm	10. xii. 30	97	1	—	124·5	0·377	0·34	100 : 88	2·7
	43		Green to green-yellow. Slight wilting and superficial scald	22. i. 31	98¾	1	—	134·5	0·379	0·39	100 : 97	3·0
Season 1931–32:												
	72	Bramley's Seedling	Green. Hard. Very young	1. vii. 31	97¾	11	238·5	233·0	0·714	0·43	100 : 58	2·2
	78		,,	17. vii. 31	95		200·0	192·0	0·667	0·60	100 : 93	2·7
	85		,,	2. ix. 31	99	2	272·5	271·5	0·455	0·40	100 : 83	2·6
	102		Very green	11. xi. 31	98½	1	223·0	218·0	0·592	0·50	100 : 80	2·6
	117		Green	12. i. 32	99	1	187·5	185·5	0·456	0·32	100 : 67	2·4
	134		Green-yellow	6. iii. 32	97½	1	138·0	134·5	0·631	0·50	100 : 76	2·6
Season 1932–33:												
	142	Bramley's Seedling	Small and young	16. viii. 32	120	4	247·0	247·0	0·252	0·25	100 : 94	2·9
Not plotted III	*a*	Bramley's Seedling	Green	24. xi. 32	95½	2	260·5	257·5	0·408	0·36	100 : 85	2·7
	b		,,	24. xi. 32	95½	2	265·0	260·5	0·362	0·33	100 : 86	2·7
	c		,,	24. xi. 32	95½	2	262·5	257·5	0·396	0·36	100 : 92	2·8

* The ratios given in column 11 are for the percentage CO_2 produced in unit time, and expressed as molecular equivalents/the percentage increase in the alcohol number in unit time, also expressed as molecular equivalents. It is of interest to note that the ratio ethyl alcohol/acetaldehyde was never less than 75/1. Since the present research is concerned only with the alcohol number and was not undertaken to determine this ratio, the alcohol numbers in the tables are not divided between ethyl alcohol and acetaldehyde.

† Blackman [1928] prefers to summarise the reaction as a quotient, glycolysis in carbon units/N.R. in carbon units. A column has been added (12) to permit of comparison with his results, should this be desired later. Each figure in this column represents the sum of the carbon equivalents of CO_2 (column 9) and of ethyl alcohol (column 10) divided by the carbon equivalent of CO_2 (column 9). No further mention will be made of this column here.

Results and Conclusions.

From the data for the alcohol number in column 10 of Table I, and the graphs of these data in Figs. 1 and 2, we see that in both Bramley's Seedling and Newton Wonder apples the average rate of anaerobic zymasis over a period of 100 hours appears to fall sharply from the value for samples of growing apples in late July to that for fully grown green apples. These changes for growing apples will be further investigated in the future. Our results for fully grown stored apples suggest that during the early stage of storage the rate of anaerobic zymasis changes but little. When, however, the colour change from green to yellow sets in, the rate of anaerobic zymasis appears to be accelerated.

Apparently the CO_2 output of these two varieties under anaerobic conditions fluctuates seasonally in a similar fashion (column 9, Table I, and Figs. 1 and 2). Thus the average rate of CO_2 output during a period of 100 hours of anaerobiosis falls sharply during the later phases of growth to a value which does not change significantly during the early phases of storage. The graphs suggest that the rate of CO_2 output by apples under anaerobic conditions increases as they turn yellow. If a connection is assumed to exist between anaerobic and aerobic processes, this rise, and also the fall during late orchard life, is what would be expected from the published results of other workers for the CO_2 output of apples in air [see Hardy, 1933].

The most important deduction to be made from the present data is that the ratio CO_2/alcohol number (Table I, column 11) does not alter significantly with the season in spite of these seasonal changes in the rates of anaerobic zymasis (Table I, column 10), and anaerobic CO_2 output (Table I, column 9). It appears, therefore, that these two magnitudes fluctuate proportionally, and this conclusion is borne out by the parallelism of the courses followed by the graphs for the alcohol number and CO_2-output in Figs. 1 and 2.

Table II. *Mean values for CO_2/alcohol number in stored Newton Wonder and Bramley's Seedling apples.*

Variety	Season	Mean value of CO_2/alcohol number	Deviation from mean	No. of measurements from which mean value is calculated
Newton Wonder	1929–30	100 : 88·5	± 2·5	2
	1930–31	100 : 89	+10 / − 5	4
	1931–32	100 : 87	± 6	3
	1932–33	100 : 65·5	+ 8 / − 5	6
	1929–30–31–32–33*	100 : 72·5	+27 / −16	15
Bramley's Seedling	1930–31	100 : 92	± 5	3
	1931–32	100 : 72	+ 8 / − 5	3
	1932–33	100 : 87·5	+ 4·5 / − 2·5	3
	1930–31–32–33*	100 : 84·5	+13 / −17	9

* See Introduction, and also Thomas and Fidler [1933].

This substantiates the important assumption implicit in Blackman's paper that the CO_2/alcohol ratio remained constant during the period of storage in which his experiments were carried out. Considerable interest centres, therefore, in the mean value of this ratio for a given season.

The data in the above table show that, except in Newton Wonder apples in seasons 1929–32, there is a different mean value for the CO_2/alcohol number ratio in each season. The difference in the mean value of this ratio for different seasons may be accidental, but experiments with large samples would be needed to settle this point.

From two observations on leaves of *Acer platanoides*, Kostytschew [1913] inferred that the ratio CO_2/ethyl alcohol fluctuates with the stage of development of the tissue. But in the present experiments the fluctuations, though considerable, were not regular. They might be attributed to a combination of experimental error, the occurrence of physiological races within the varieties, and the use of small samples. Probably the last is the most important cause; for, as will be shown in a later paper, this sampling error is much larger than the maximum error encountered in the estimation of ethyl alcohol and acetaldehyde in apples.

The average value for the CO_2/alcohol number ratio in Bramley's Seedling and Newton Wonder apples may be placed at 100/80–85. We have, however, obtained some evidence (see Appendix) of varietal differences in regard to the ratios.

As the CO_2/alcohol number ratio in molecular equivalents is not unity, as demanded by the usual equation for alcoholic fermentation, $C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O$, Blackman's method of evaluating glycolysis must be slightly modified. He assumes that the ratio is unity, and he calculates glycolysis from CO_2 output in nitrogen by multiplying this figure by 3. If, however, the CO_2/alcohol number ratio is 100/85, only 1·7 atomic proportions of carbon would be lost as ethyl alcohol for each one lost as CO_2, so that glycolysis would be 2·7 times the CO_2 output in nitrogen. In view of the fluctuations about a mean value of 100/85, the CO_2/alcohol number ratio should be measured in a sample of the same fruit before attempting to estimate glycolysis. Many other examples are known of CO_2/ethyl alcohol ratios in which CO_2 predominates, and only rarely has a ratio of unity been reported and confirmed. Thus Boysen-Jensen [1922] for grapes and carrots and Kostytschew [1913] for many tissues including apples obtained ratios ranging from 100/40 to 100/80. Both conclude that anaerobic respiration in higher plants is a mixed reaction which consists only in part of alcoholic fermentation, the accompanying reaction being independent of glycolysis.

This latter conclusion cannot be accepted unconditionally, for products of zymasic cleavage not estimated by the present method may account for the low ratios obtained. Acetaldehyde, however, cannot be one of these, since this compound is included in the alcohol number.

Summary.

1. Zymasis in nitrogen follows the same seasonal curve as CO_2 output in air (O.R.).

2. In any one season, the ratio CO_2/alcohol number (which is practically identical with the ratio CO_2/ethyl alcohol) fluctuates widely about a mean value, but this mean does not alter as apples age.

3. The mean value for this ratio in Newton Wonder and Bramley's Seedling apples during three seasons is of the order 100/80–85.

4. The mean value for the CO_2/alcohol number ratio may vary from season to season.

5. The results of preliminary experiments suggest that there are great varietal differences in the CO_2/alcohol number ratio; this ratio fluctuates between 100/47 to 100/92 in small samples of different varieties of ripe apples.

In addition to the acknowledgments which have already been made [Fidler, 1933], I wish to thank the Armstrong College Research Committee for a grant for the purchase of nitrogen.

APPENDIX.

A SURVEY OF CO_2/ALCOHOL NUMBER IN SEVERAL VARIETIES OF APPLES.

The following is an account of a short survey of CO_2/alcohol number in several varieties of ripe apples purchased from local fruiterers. Estimations were carried out precisely as described in the main paper.

Variety	Country of origin	Date of commencement of exp.	Duration of exposure to nitrogen (hours)	g. CO_2 per 100 g. fruit per 100 hours	Increase in the % alcohol number in 100 hours (corrected for control values)	CO_2/alcohol number
Dougherty	Australia	23. viii. 32	120	0·169	0·14	100 : 81
Gravenstein	U.S.A.	23. viii. 32	120	0·277	0·19	100 : 65
Sturmer	Australia	23. viii. 32	120	0·327	0·26	100 : 77
Keswick	England	1. ix. 32	120	0·264	0·16	100 : 57
Beauty of Bath	England	1. ix. 32	120	0·209	0·13	100 : 61
Belflower	France	1. ix. 32	120	0·212	0·15	100 : 68
Maiden's Blush	U.S.A.	28. ix. 32	119½	0·289	0·22	100 : 73
Wealthy	U.S.A.	28. ix. 32	119½	0·167	0·08	100 : 47
Jonathan	U.S.A.	11. x. 32	120½	0·151	0·08	100 : 54
McIntosh Red	Canada	11. x. 32	120½	0·193	0·17	100 : 85
Cleopatra	Tasmania	11. vii. 30	99½	0·164	0·16	100 : 93
Cleopatra	Tasmania	6. v. 31	98¾	0·255	0·21	100 : 85
Newtown Pippin	U.S.A.	8. iii. 33	114½	0·113	0·10	100 : 90
Newtown Pippin	U.S.A.	25. iv. 33	148¾	0·177	0·15	100 : 81
Granny Smith	Australia	9. vi. 33	49	0·278	0·20	100 : 65

It appears that as for Bramley's Seedling and Newton Wonder apples, so also for these other varieties, the ratio CO_2/alcohol number is always higher than that required by the usual equation for alcoholic fermentation. As small samples were used, the ratios given in the table cannot be taken as true means, but the differences shown suggest that the mean ratios may possibly vary greatly in different varieties of apples.

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CCXXII. STUDIES IN ZYMASIS.

VI. ZYMASIS BY APPLES IN RELATION TO OXYGEN CONCENTRATION.

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(*Received July 26th, 1933.*)

The ensuing subject matter deals principally with the problem of determining the concentration of oxygen at which the anaerobic respiration of apples becomes extinguished. Pure nitrogen has been used in most of the recent work on the anaerobic respiration of plants, and we propose to follow Blackman [1928] and Parija [1928] in describing the CO_2 component of anaerobic respiration as nitrogen respiration (N.R.). We shall refer to the concentration of oxygen that extinguishes N.R. (*i.e.* anaerobic respiration) as the extinction point of N.R.

Bramley's Seedling apple was selected as a subject for experiment because much important work has been performed on this variety at the Botany School and at the Low Temperature Station at Cambridge; but experiments have also been performed with the Newton Wonder apple.

The scope of this research was widened to test Blackman's contention (see below) that, even for a single variety for a given season, the extinction point of N.R. is not a fixed magnitude throughout the storage period but shifts to higher values as the fruit ages. The extinction points of N.R. for Newton Wonder and Bramley's Seedling apples have therefore been determined late as well as early in the storage season. Further, as our experiments extended over 2 years, we can consider whether seasonal factors affect the time at which the shift of the extinction point begins in stored fruit.

The novelty of our work resides essentially in the method adopted for finding the extinction point for N.R. The method is based on the facts, established by earlier quantitative researches [Thomas, 1925], first that healthy apples do not normally undergo zymasis in air, and secondly that under anaerobic conditions ethyl alcohol accumulates in the flesh tissue until this is dead. It is argued that zymasis probably also occurs in low concentrations of oxygen, and that there is for any apple in a given physiological state a limiting concentration of oxygen that extinguishes this process.

By our methods of analysis, ethyl alcohol, acetaldehyde (which also accumulates in small amounts) and possibly traces of other compounds which are volatile in steam, are measured together to give what we term the "alcohol number" [see Fidler, 1933, 1]. This number may be taken as a measure of the zymasic products which have accumulated in the flesh tissue. Under anaerobic conditions CO_2 (Blackman's N.R.) is simultaneously produced, and there is evidence for Bramley's Seedling and Newton Wonder apples that the greater part of this N.R. is due to zymasis, and that the ratio alcohol number/N.R. for a given variety is constant during a single season [Fidler, 1933, 2]. From

this constancy we infer that the alcohol number may also be taken as a measure of N.R., and that the extinction point of N.R. will be that concentration of oxygen at which the alcohol number becomes zero.

Earlier workers by other methods have aimed at determining the extinction point of N.R. Thus Stich [1891], and several workers quoted by him, measured the effect of lowered oxygen concentration on the respiratory quotient of certain plant tissues, the general principle being that this quotient is increased when zymasis (*i.e.* N.R.) begins. None of these workers, however, measured alcohol.

Blackman [1928] inferred from CO_2 output curves that the extinction point of N.R. for Bramley's Seedling apples is below 5 % in early storage, and that it increases as the apples age. Again, no measurements of alcohol were made. The results we have obtained by using the alcohol number as an index are unambiguous, and they permit us to test whether the arguments based on data collected by other methods are valid. In order to compare the behaviour of the apples used by us with the behaviour of those used by Blackman and Parija [1928], we have also measured the rate of CO_2 production.

So far as we know, this is the first time that CO_2 and the alcohol number (or any other measure of accumulating zymasic products) have been simultaneously determined in gas mixtures of nitrogen and oxygen. Some of the advantages accruing from this procedure are developed later (see Section III). Thus, even when N.R. forms one component of the total CO_2 produced, what has been termed the total carbon loss [Blackman, 1928] has been calculated. Further, it is also possible by calculation to distribute total CO_2 between N.R. and normal respiration (Blackman's O.R.). The results of a few calculations are given in Section II in Table II, but we have not yet sufficient data for critical discussion. In fine, we consider that no analysis of glycolysis in which N.R. CO_2 is a product can approach completion unless cognisance is taken of the accumulating products of zymasis, should it be known that this process occurs under anaerobic conditions.

I. Materials and methods.

An account has already been given [Fidler, 1933, 1] of the times of gathering, and of the modes of storing and dispatching the fruit used in the experiments we are about to describe. The apparatus and methods of analysis employed have also been described by Fidler [1933, 1, 2].

Upon receiving the apples from storage the CO_2 output in air at 23° was determined. The air stream was then changed for one of pure nitrogen or of a nitrogen-oxygen mixture[1] at the rate of 3 litres per hour when the sample was a single apple, or 6 litres per hour when larger samples were used. Analyses of the issuing gas showed that the composition of the gas stream at this speed was not appreciably altered by the respiration of the fruit. Exposure to the artificial atmosphere lasted for about 4 days.

Sets of experiments were carried out at intervals during the storage season, the apples used in any one set being roughly of the same age. Each set consisted of two groups of four separate experiments, each of which was performed in a different concentration of oxygen. Six weeks to 2 months elapsed between each set of experiments. Thus, in addition to showing the effect of varying oxygen concentrations on apples of roughly the same age, it was also possible to deter-

[1] The nitrogen-oxygen mixtures were obtained in cylinders from the British Oxygen Company, Ltd., Walker Gate, Newcastle-on-Tyne, their composition being determined accurately by means of a Haldane gas analysis apparatus before use.

mine the effect of increasing age on zymasis by the fruit in any given concentration of oxygen. Work on a set of experiments lasted about 2 weeks, a new consignment of fruit for the second group of four experiments being timed to arrive on the day the apples used in the first four were distilled.

II. Results.

A. *The alcohol number.*

Preliminary work [Fidler, 1933, 1] showed that the alcohol number of storage controls was not the best basis for evaluating change in the alcohol number owing to oxygen shortage; for it appears that zymasis may be induced by other factors connected with the experimental conditions. For gas mixtures containing oxygen, interest resides chiefly in the figures in column 11 of Table I. These have been obtained by deducting the corresponding conditions control values [Fidler, 1933, 1, Table II], from the alcohol numbers[1] found by analysis. There is no evidence that conditions as such increase zymasis in pure nitrogen, and for this gas the storage control has been used.

B. *The rate of CO_2 production, distributed between O.R. and N.R. Carbon loss.*

Only the results for the rate of production of CO_2 by Bramley's Seedling apples in 1931–2 will be considered here[2].

In Section III we compare our results for CO_2 output with those obtained by Parija [1928] who used Bramley's Seedling apples from a different locality. Our experiments differ from his in one important respect. To investigate the extinction point of N.R. he used the same apple in different concentrations of oxygen. Since we determined the alcohol number as well as the CO_2 output, we necessarily had to use different apples for every experiment.

Notes to Table I.

(*a*) *Weight of apple.* Up to experiment 62, the final weight was used in calculating the percentage concentration of products of zymasis. After this time the initial weight has been used. The loss in weight was never more than 6 % in a sound apple and was usually of the order of 1 to 3 %. As all the numbers in any series have been calculated to the same basis, the error is not serious.

(*b*) *Exps.* 106, 107 *and* 108 *on Bramley's Seedlings* were allowed to continue for about 8 days and then analysed. The alcohol numbers given in the table have been calculated by dividing the alcohol numbers obtained in the analysis by the quotient: CO_2 output per 8 days/CO_2 output during the first 100 hours.

(*c*) The results given for experiments in pure nitrogen are taken from Fidler [1933, 2], and are included for the sake of comparison with those obtained in 0·8 % oxygen.

(*d*) The storage control has been used in experiments where the alcohol number was greater than that in a conditions control.

[1] The ratio alcohol/acetaldehyde in these experiments was intermediate between 45/1 (the highest recorded in air) and 75/1 (the lowest recorded in nitrogen). It is not proposed to analyse these figures further since the research was not planned to determine the effect of oxygen tension and age of apples on this ratio. It is proposed, however, to carry out experiments to test these points in the near future.

[2] Results for Bramley's Seedlings in another season and for Newton Wonders in three seasons are given in a Ph.D. thesis [Fidler, 1932], where they have been treated in the manner adopted here for this selected set of results for Bramley's Seedlings.

Table I. *Changes in the percentage alcohol number for Newton Wonder and Bramley's Seedling apples in nitrogen-oxygen mixtures at 23°. (See notes to Table I.)*

No. of exp.	Variety of fruit	State of fruit	Date exp. begins	Hours' exposure to gas	Percentage oxygen in gas	No. of apples in sample	Wt. of sample (g.)		The percentage alcohol number after 100 hours	
							Initial	Final	After deduction of storage control value	After deduction of conditions control value
1	2	3	4	5	6	7	8	9	10	11
Season 1930–31. From orchard										
15	Newton Wonder	Small. Green. Very young	22. vii. 30	123½	5·7	5	—	152·0	0·002	—
From cool storage										
17	Newton	Green. Hard	24. ix. 30	97½	Nil	1	—	112·5	0·19	—
18	Wonder	Green. Hard	24. ix. 30	97½	3·6	1	—	93·5	—	Nil
19		,,	24. ix. 30	99¾	5·7	1	—	113·5	—	Nil
20		Green. Hard. Young	1. x. 30	142	21·0	1	—	100·5	0·01	—
30		Green to green-yellow	27. xi. 30	97	Nil	1	—	193·0	0·25	—
31		Green-yellow	27. xi. 30	97	2·6	1	—	200·5	0·14	0·14
32		,,	27. xi. 30	99½	3·6	1	—	200·0	0·08	0·08
37		Yellow. Slightly wilted	8. i. 31	99½	Nil	1	—	105·0	0·35	—
38		Green-yellow. Slightly wilted	8. i. 31	99½	2·6	1	—	142·5	0·28	0·27
39		,,	8. i. 31	99½	3·6	1	—	139·5	0·25	0·23
Season 1931–2. From orchard										
73	Newton	Green. Hard. Very young	1. vii. 31	97¾	Nil	12	103·0	96·5	0·70	—
74	Wonder	Green. Hard. Very young	1. vii. 31	97¾	0·8	12	102·0	98·5	0·67	0·65
C		Green. Hard. Very young	17. vii. 31	113	21·0	6	134·5	132·5	0·01	—
79		Green. Hard. Very young	17. vii. 31	95	Nil	9	174·5	167·0	0·73	—
80		,,	17. vii. 31	72½	0·8	8	178·5	174·5	0·63	0·60
From cool storage										
81	Newton	Green. Hard. Very young	26. viii. 31	98½	Nil	3	208·0	201·0	0·38	—
82	Wonder	,,	26. viii. 31	98½	3·0	3	213·0	212·5	—	Nil
83		,,	26. viii. 31	98½	5·0	3	203·0	198·0	—	Nil
84		Green. Hard. Very young	26. viii. 31	122	21·0	3	212·0	207·0	0·02	—
89		Green. Hard. Very young	10. ix. 31	99½	9·5	2	213·0	209·5	—	Nil
90		,,	10. ix. 31	99½	40·0	3	207·5	203·5	—	Nil
91		,,	10. ix. 31	99½	100	3	200·0	194·5	—	Nil
95		Green	28. x. 31	99	Nil	2	252·5	246·0	0·32	—
96		,,	28. x. 31	99	3·0	2	288·0	284·0	—	Nil
97		,,	28. x. 31	99	100	2	259·0	254·5	—	Nil
98		,,	28. x. 31	140	21·0	2	321·0	312·5	0·01	—
99		Green to yellow-green	4. xi. 31	98½	8·0	2	329·0	324·0	—	Nil
100		,,	4. xi. 31	98½	15·2	2	336·0	331·5	—	Nil
101		Yellow-green	4. xi. 31	98½	28·0	2	286·0	285·5	—	Nil
109		Green to yellow-green	10. xii. 31	98½	Nil	2	298·5	293·5	0·50	—
110		Yellow-green	10. xii. 31	98½	2·4	2	295·0	289·0	—	Nil
111		,,	10. xii. 31	98½	2·9	2	293·0	290·0	—	Nil
112		Green to yellow-green	10. xii. 31	121½	21·0	2	279·0	270·0	0·45	—
113		Yellow-green to green-yellow	16. xii. 31	99	8·5	2	299·0	295·5	—	Nil
114		Yellow-green	16. xii. 31	99	15·2	2	280·5	279·0	—	Nil
115		Green-yellow	16. xii. 31	99	100	2	281·0	278·5	—	Nil
116		Yellow	16. xii. 31	99	60	2	264·5	258·5	—	Nil
125		Green-yellow	27. i. 32	99	Nil	1	84·0	81·5	0·50	—
126		Green-yellow. First sign of wrinkling	27. i. 32	99	8·3	1	83·5	81·0	0·08	0·06
127		,,	27. i. 32	99	2·4	1	74·5	72·0	0·07	0·05
128		,,	27. i. 32	140½	21·0	1	97·5	97·0	0·02	—
129		,,	10. ii. 32	98	2·9	1	106·5	105·0	0·17	0·11
130		,,	10. ii. 32	98	5·3	1	92·5	91·5	0·19	0·13
131		,,	24. ii. 32	96¼	60·0	1	86·5	84·5	0·02	0·02
133		,,	24. ii. 32	138	21·0	1	87·0	83·5	0·02	—

130		,,	10. ii. 32	98	2·9	1	106·5	105·0	0·17	0·11
131		,,	10. ii. 32	98	5·3	1	92·5	91·5	0·19	0·13
133		,,	24. ii. 32	96¼	60·0	1	86·5	84·5	0·02	0·02
			24. ii. 32	138	21·0	1	87·0	83·5	0·02	—

Season 1930–31. From cool storage

25	Bramley's	Green. Hard. Young	19. xi. 30	97¾	Nil	1	—	148·5	0·34	—
26	Seedling	Green. Hard	19. xi. 30	97¾	2·6	1	—	147·5	0·08	0·05
27		,,	19. xi. 30	99½	5·3	1	—	149·0	0·03	0·004
34		Green. Firm	10. xii. 30	97	Nil	1	—	124·5	0·34	—
35		,,	10. xii. 30	99½	2·6	1	—	153·5	0·17	0·16
43		Green to green-yellow	22. i. 31	98¾	Nil	1	—	134·5	0·37	—
44		Green-yellow	22. i. 31	98¾	2·6	1	—	150·0	0·25	0·17
45		Green-yellow. Wilted	22. i. 31	98¾	5·0	1	—	118·5	0·18	0·10
46		Green-yellow. Very slightly wilted	29. i. 31	116	21·0	1	—	170·0	0·06	—
50		Green-yellow	5. ii. 31	99½	9·5	1	—	189·5	—	Nil
51		Yellow-green. Slightly wilted	5. ii. 31	99½	11·0	1	—	187·5	—	Nil
52		,,	5. ii. 31	99½	15·3	1	—	193·0	—	Nil
53		,,	11. ii. 31	98	28·0	1	—	142·0	—	Nil
54		Yellow-green. Badly wilted	11. ii. 31	98	100	1	—	90·0	—	Nil
55		Yellow. Badly scalded and wilted	5. iii. 31	116	21·0	1	—	147·0	0·15	—
57		,,	5. iii. 31	98	69·0	1	—	121·5	—	Nil
58		,,	5. iii. 31	98	40·0	1	—	139·5	—	Nil

Season 1931–2. From orchard

71	Bramley's	Green. Hard. Very young	1. vii. 31	97¾	0·8	11	249·0	239·5	0·45	0·44
72	Seedling	Green. Hard. Very young	1. vii. 31	97¾	Nil	11	238·5	233·0	0·43	—
75		,,	8. vii. 31	116½	21·0	8	172·0	161·5	0·01	—
77		Green. Hard. Very young	17. vii. 31	72½	0·8	5	183·5	179·0	0·58	0·57
78		Green. Hard. Very young	17. vii. 31	95	Nil	5	200·0	192·0	0·60	—
85		,,	2. ix. 31	99	Nil	2	272·5	271·5	0·40	—
86		Green. Hard. Very young	10. ix. 31	99	3·0	2	215·5	214·0	—	Nil
87		,,	2. ix. 31	99	5·0	2	236·0	235·5	—	Nil
88		Green. Hard. Very young	2. ix. 31	140½	21·0	2	218·5	218·0	0·01	—
92		Green. Hard. Very young	18. ix. 31	98	8·0	2	183·5	181·0	—	Nil
93		,,	18. ix. 31	98	40·0	2	186·0	182·0	—	Nil
94		,,	18. ix. 31	98	100	2	208·5	205·5	—	Nil

Season 1931–2. From cool storage

102	Bramley's	Green	11. xi. 31	98½	Nil	1	223·0	218·0	0·50	—
103	Seedling	,,	11. xi. 31	97	3·0	1	211·0	206·0	—	Nil
104		,,	11. xi. 31	98½	100	2	376·5	372·0	—	Nil
105		,,	11. xi. 31	115¾	21·0	1	207·5	202·0	0·05	—
106		,,	18. xi. 31	—	8·5	1	234·5	—	—	Nil
107		,,	18. xi. 31	—	28·0	1	206·5	—	0·06	0·005
108		,,	18. xi. 31	—	69·0	1	164·5	—	0·06	0·003
117		,,	12. i. 32	99	Nil	1	187·5	185·5	0·32	—
118		,,	6. i. 32	99	8·3	1	180·5	180·5	—	Nil
119		,,	6. i. 32	99	2·4	1	112·0	110·0	—	Nil
120		,,	6. i. 32	138½	21·0	1	158·0	157·5	0·06	—
121		,,	13. i. 32	99	0·8	1	181·0	177·0	0·34	0·28
122		,,	13. i. 32	99	2·9	1	191·0	191·0	—	Nil
123		,,	13. i. 32	99	5·2	1	148·0	146·0	—	Nil
124		,,	13. i. 32	99	8·5	1	172·5	172·0	—	Nil
134		Green-yellow	6. iii. 32	97½	Nil	1	138·0	134·5	0·50	—
135		,,	16. iii. 32	97½	8·3	1	247·5	244·0	—	Nil
136		,,	16. iii. 32	97½	2·4	1	160·5	157·5	0·10	0·02
137		,,	16. iii. 32	140	21·0	1	167·5	164·0	0·09	—
138		Green-yellow. First sign of wrinkling	31. iii. 32	97¾	2·9	1	99·5	97·5	0·11	0·07
139		,,	31. iii. 32	97¾	5·3	1	160·5	158·0	0·05	0·004
140		,,	31. iii. 32	97¾	60·0	1	152·0	150·5	—	Nil
141		,,	31. iii. 32	116½	21·0	1	127·5	126·0	0·05	—

Table II. *Rate of CO_2-production, N.R., O.R., carbon loss, and CO_2/alcohol number of Bramley's Seedling apples during 1931–2. (For details of state of fruit, date of experiment and the alcohol number, see Table I.)*

		g./100 g. apple/100 hours						
				Corrected for conditions control values†				
Exp. No.	Oxygen in gas %	Actual carbon loss*	Actual CO_2 output	CO_2	N.R.	O.R.	CO_2/alcohol number	Total carbon loss / Carbon lost as CO_2
1	2	3	4	5	6	7	8	9
From orchard:								
71	0·8	0·42	0·680	0·67	0·50	0·17	100 : 63	2·3
72	Nil	0·42	0·714	—	0·71	Nil	100 : 58	2·2
75	21·0	0·15	0·542	0·54	—	0·54	—	1·0
77	0·8	0·50	0·727	0·72	0·64	0·07	100 : 76	2·6
78	Nil	0·50	0·667	—	0·67	Nil	100 : 93	2·7
85	Nil	0·33	0·455	—	0·46	Nil	100 : 83	2·6
86	3·0	0·05	0·176	0·16	Nil	0·16	—	1·0
87	5·0	0·07	0·239	0·22	Nil	0·22	—	1·0
88	21·0	0·13	0·446	0·43	—	0·43	—	1·0
92	8·0	0·06	0·200	0·19	Nil	0·19	—	1·0
93	40·0	0·06	0·203	0·19	Nil	0·19	—	1·0
94	100	0·10	0·337	0·32	Nil	0·32	—	1·0
From cool storage:								
102	Nil	0·46	0·592	—	0·59	Nil	100 : 80	2·6
103	3·0	0·14	0·390	0·33	Nil	0·33	—	1·0
104	100	0·18	0·563	0·50	Nil	0·50	—	1·0
105	21·0	0·18	0·545	0·49	—	0·49	—	1·0
106	8·5	0·16	0·477	0·42	Nil	0·42	—	1·0
107	28·0	0·17	0·469	0·41	0·005	0·41	100 : 3	1·0
108	69·0	0·20	0·605	0·55	0·004	0·54	100 : 1	1·0
117	Nil	0·30	0·456	—	0·46	Nil	100 : 67	2·4
118	8·3	0·15	0·419	0·35	Nil	0·35	—	1·0
119	2·4	0·15	0·414	0·35	Nil	0·35	—	1·0
120	21·0	0·20	0·594	0·54	—	0·54	—	1·0
121	0·8	0·33	0·520	0·46	0·46	Nil	100 : 58	2·2
122	2·9	0·14	0·384	0·32	Nil	0·32	—	1·0
123	5·2	0·17	0·465	0·40	Nil	0·40	—	1·0
124	8·5	0·19	0·539	0·47	Nil	0·47	—	1·0
134	Nil	0·45	0·631	—	0·63	Nil	100 : 76	2·6
135	8·3	0·18	0·496	0·44	Nil	0·44	—	1·0
136	2·4	0·20	0·490	0·42	0·02	0·40	100 : 4	1·0
137	21·0	0·21	0·567	0·57	—	0·52	—	1·0
138	2·9	0·22	0·499	0·44	0·09	0·36	100 : 14	1·6
139	5·3	0·18	0·493	0·44	0·006	0·43	100 : 1	1·0
140	60·0	0·22	0·615	0·57	Nil	0·57	—	1·0
141	21·0	0·22	0·638	0·59	—	0·59	—	1·0

* *I.e.* total zymasis in carbon units (carbon equivalent of column 4, *plus* the carbon equivalent of the conditions control alcohol number).

† Except in Exps. 72, 78, 85, 102, 117, 134, where O_2 % = Nil.

Notes to Table II.

(*a*) *Distribution of CO_2 between O.R. and N.R.* Before distributing the CO_2 given off in gas mixtures of nitrogen and oxygen between O.R. and N.R., allowance must be made for the CO_2 component of zymasis which is induced by conditions as such (see p. 1631). This part of the total CO_2 produced may be calculated from the alcohol number of the conditions control.

For this calculation, instead of using the conventional equation for alcoholic fermentation, we prefer to use the formula experimentally determined for the variety of apple under observation. Strictly, this formula should be determined each season, but there are insufficient data for this refinement. Instead we take the mean value for the given variety for all the seasons during which

this apple has been used. Thus the preliminary work over two seasons [Fidler, 1933, 2] permits us to write for Bramley's Seedlings: respirable substrate → 100 CO_2 + 85 C_2H_6O. By subtracting this CO_2 component of zymasis occurring in air at 23° under experimental conditions from the total CO_2 absorbed by the baryta, we arrive at a number representing the CO_2 which must be distributed between O.R. and N.R.

If the alcohol number obtained in any experiment is of the same order as that of a conditions control, we conclude that there is no N.R. due to oxygen shortage. All the CO_2 produced is then attributed to O.R.

When, however, zymasis due to oxygen shortage is detected, the CO_2 component is calculated as we have described above. Thus, for Bramley's Seedlings, in calculating N.R. due to oxygen shortage, 100 g.-mol. equivalents of CO_2 are taken as equivalent to 85 g.-mol. equivalents of zymasic products accumulating in the tissues. The O.R. occurring simultaneously may be found by subtracting the calculated value for N.R. from the total CO_2 output corrected for experimental conditions.

(*b*) *Calculation of the percentage carbon loss.* The percentage carbon loss, given in Table II, is the sum of the weights in g. of carbon given off as CO_2 and excreted in the tissues as ethyl alcohol and acetaldehyde.

(*c*) *The quotient Total carbon loss/Carbon lost as CO_2.*

In the paper dealing with zymasis in nitrogen [Fidler, 1933, 2], a column was included giving Glycolysis in C units/N.R. in C units. Such a column cannot be included in the present paper, since total carbon loss in nitrogen-oxygen mixtures is not equal to glycolysis in carbon units. The difference is due to O.A. (oxidative anabolism [Blackman, 1928]). This, however, cannot be evaluated from our data. A column has been added here giving the quotient Total carbon loss/Carbon lost as CO_2. When there is no N.R. the value of this quotient is unity. As N.R. forms an increasingly important component of respiration, the quotient rises to a maximum of 2·7 (for an anaerobic CO_2/alcohol ratio of 100/85).

III. Discussion and general conclusions.

(A) *The effect of varying oxygen concentrations on the alcohol number in Newton Wonder and Bramley's Seedling apples of different ages and in different seasons.*

(i) *The alcohol number as a measure of N.R., and the possible relations between N.R. and oxidation processes.* The graphs in Figs. 1*a* and 1*b*, and 2*a* and 2*b*[1], in which are represented the results given in Table I for Newton Wonder and Bramley's Seedling apples, show the concentrations of oxygen that extinguish N.R. at different times in the storage season, and what is the relative extent of N.R. when it occurs. When the alcohol number points lie above the *x*-axis, zymasis takes place to a greater extent than in a conditions control [see Fidler, 1933, 1]. It follows first that N.R. then forms one component of the respiratory process, and, secondly, that this N.R. occurs owing to a deficiency of oxygen in the gas mixture passed over the fruit. Further, the relative magnitudes of N.R. in the total respiration in different gas mixtures[2] may, of course, be

[1] To avoid crowding of graphs we have assembled in Figs. 1*a* and 1*b* the results for apples before they became pronouncedly yellow, and in Figs. 2*a* and 2*b* the results for the same populations when they were rapidly becoming or had become yellow. This colour change criterion of the ageing of the flesh tissue of apples has been used before [see *e.g.* Blackman and Parija, 1928]. Evidence given later in this section suggests, however, that other phenomena of ageing do not necessarily march in line with this colour change, *i.e.* apples of the same variety, from the same population and of the same colour are not necessarily in the same physiological state in all respects.

[2] In column 9, Table II in Section II, it will be seen that the ratio Total carbon loss/Carbon lost as CO_2 decreases from 2·7 to unity when the oxygen concentration is increased. The extinction point is the minimum oxygen concentration at which this ratio becomes unity.

judged from the differences in the heights of the points above the x-axis. When, however, the alcohol number points lie on the x-axis, there is no zymasis beyond that occurring in a conditions control. In these cases there is sufficient oxygen in the gas mixtures to extinguish N.R. completely.

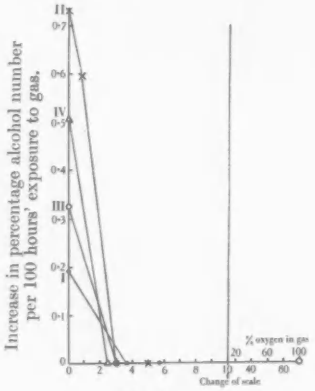

Fig. 1*a*.

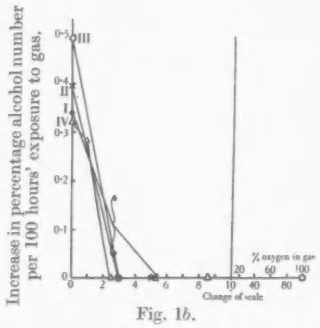

Fig. 1*b*.

Fig. 1*a*. Percentage alcohol numbers of green to yellow-green Newton Wonder apples.

Season 1930–1. From store: I, 24. ix. 30. ●
Season 1931–2. From orchard: II, 17. vii. 31–26. viii. 31. ×
From store: III, 28. x. 31. ○
From store: IV, 10. xii. 31. △

Fig. 1*b*. Percentage alcohol numbers of stored green to yellow-green Bramley's Seedling apples.

Season 1930–1. I, 19. xi. 30–10. xii. 30. ●
Season 1931–2. II, 2–10. ix. 31. ×
III, 11. xi. 31. ○
IV, 6–13. i. 32. △

The graphs afford unquestionable evidence that at all times in the storage season, as the oxygen concentration outside the apples is increased, N.R. gradually falls from the maximum value attained in pure nitrogen. In most of our experiments this fall in N.R. continued until zymasis completely ceased: then, of course, the extinction point of N.R. is the minimum oxygen percentage at which the alcohol number reaches zero. This percentage is not the same for all apples (see later sub-sections).

We have insufficient data for low concentrations of oxygen to calculate the changing quantitative relations between N.R. and oxidation processes as the oxygen concentration rises (see also footnote on p. 1640). The most obvious hypothesis is that N.R. is substituted step by step by oxidation processes in rising oxygen concentrations. There are two conflicting contemporary views that would then have to be borne in mind in explaining the inhibitory action of oxygen on zymasis. According to one view, N.R. and oxidative processes are cognate. Accepting this view, we might infer from the experiments and arguments of Meyerhof [1925] and Blackman [1928] that, as nitrogen is progressively replaced by oxygen, the products of glycolysis are diverted from ethyl alcohol and CO_2 (N.R.) to water and CO_2 (O.R.) and O.A. (oxidative anabolism,

or the Meyerhof-Pasteur reaction). According, however, to the alternative view (see for example Lundsgaard [1930] and Boysen-Jensen [1922; 1931], the enzyme systems governing N.R. and oxidation processes are quite distinct. Should this view become established it would mean that the contiguous processes of N.R. and oxidation occurring in low concentrations of oxygen are unconnected although they affect the same substrate. It would then follow

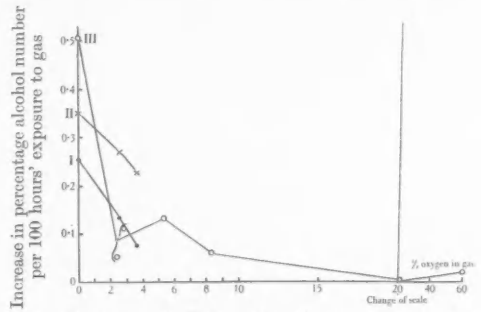

Fig. 2*a*. Percentage alcohol numbers of green-yellow to yellow Newton Wonder apples.

Season 1930–1. I, 27. xi. 30. ●
II, 8. i. 31. ×
Season 1931–2. III, 27. i. 32–24. ii. 32. ○

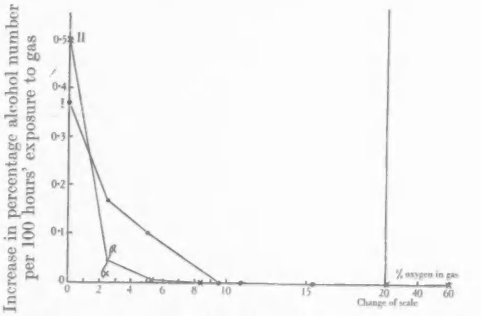

Fig. 2*b*. Percentage alcohol numbers of green-yellow to yellow Bramley's Seedling apples.

Season 1930–1. I, 22. i. 31–5. ii. 31. ●
Season 1931–2. II, 16–31. iii. 32. ×

that in rising oxygen concentrations zymasis is increasingly inhibited and that simultaneously oxidation processes, which are independent of anaerobic glycolysis, are activated to an equivalent extent.

(ii) *The extinction point of N.R. early in the storage season.* In Fig. 1*a* are recorded the alcohol numbers for Newton Wonder apples during two seasons at the beginning of the cool storage periods. When these experiments were started we thought that zymasis would occur in all concentrations of oxygen

less than 5 %. But our results for this variety indicate that early in storage zymasis, owing to oxygen shortage, does not occur even in 2·5 % oxygen[1]. For Bramley's Seedlings, in 1931–2, it will be seen from graphs II, III and IV in Fig. 1*b*, that this same concentration of oxygen completely inhibited zymasis in September and November 1931, and even as late as January 1932. At this last date the apples which we used were yellow-green[2]. Our results for this same variety at the corresponding time in the previous season cannot be used in the present analysis; for before the middle of December 1930 (see graph I, Fig. 1*b*) the apples, whilst still green, underwent zymasis in all gas mixtures containing less than 5 % of oxygen. It is probable, however, that had we experimented on Bramley's Seedlings at an earlier date in the autumn of 1930, zymasis would have been stopped by a lower concentration of oxygen (see next subsection).

We conclude, therefore, that the extinction point of N.R. for apples of these two varieties whilst they are still on the tree or early in storage is not greater than 2·5 % oxygen. This is a surprisingly low figure. And it must be remembered that in a bulky organ, such as an apple, the internal concentration of oxygen in the intercellular spaces around the respiring cells is always less than the concentration of oxygen outside. Questions concerning the oxygen relations of possible respiratory systems in cells suggest themselves, but must for the present be left unanswered.

(iii) *The shift of the extinction point in ageing apples.* The graphs in Figs. 2*a* and 2*b* show that zymasis is not completely inhibited by 2·5 % oxygen when senescent characteristics, such as the change to green-yellow and yellow, wilting and superficial scald, appear. Thus we conclude that although the extinction point of N.R. appears to be constant early in the storage season, it shifts to higher values when signs of ageing become more marked.

The charts for the preliminary experiments on green-yellow Newton Wonder apples in 1930–1 (Fig. 2*a*, graphs I and II) show that, notwithstanding the steep decline in the alcohol numbers with increasing oxygen concentrations, zymasic products accumulated in apples in 3 % oxygen in greater amounts than in the conditions controls. Early in 1932 experiments were carried out with a wider range of gas mixtures and we interpret graph III as showing that after January, in green-yellow air-stored Newton Wonder apples, (*a*) N.R. is not completely extinguished in any concentration of oxygen (*cf.* the complete extinction of N.R. in 3 % oxygen in December 1931—graph IV, Fig. 1*a*) and (*b*) for each increase in oxygen concentration up to a value that is less than 5 % oxygen, the corresponding decrease in N.R. is more rapid than when the oxygen concentration is increased beyond 5 %. We are not prepared, from this experiment alone, to state what is the precise form of the curve representing this fall in N.R. in senescent apples.

[1] Graph II in Fig. 1*a* shows that very young green apples picked from orchard trees behave similarly to fully grown green apples taken from store.

[2] Although colour is a useful guide for grouping apples, it is clearly not a rigid criterion of physiological state. Here we see that zymasis occurred in green apples in December 1930 in 2·5 % oxygen but was completely inhibited by this concentration in yellow-green apples in the next season. On a colour standard these latter apples would be considered to have been the more senescent of these two samples, but in terms of the respiratory events in the cells of the flesh tissue we consider that they were the less senescent. There is an element of hazard at present in arranging comparable experiments for stored apples in two different seasons. This might conceivably be lessened by using in successive seasons only those apples that have approximately the same extinction point of N.R.

In the comparable experiments with Bramley's Seedling apples (Fig. 2*b*), we note from graph I that in late January to early February 1931 as much as 9 % oxygen was needed to extinguish N.R. But when we examine graph I in Fig. 1*b*, we see that in December 1930, *i.e.* about 6 weeks earlier in the storage season, zymasis was nearly completely checked by only 5 % oxygen. For Bramley's Seedling apples ageing in air-stores during 1930–1, as well as for Newton Wonder apples in this same season, there is clear evidence, therefore, that in late storage greater concentrations of oxygen are needed to inhibit zymasis, *i.e.* to extinguish N.R., than in early storage.

In 1931–2, the results for Bramley's Seedlings, although not so striking, point to the same conclusion. Thus graphs II, III and IV in Fig. 1*b* show quite definitely that there was no zymasis in concentrations greater than 3 % oxygen before January 1932; and from graph II in Fig. 2*b* we see that by March 1932 the alcohol number had risen distinctly above the conditions control value in 3 % oxygen. At this latter date the slight rise shown in 5·3 % oxygen is by itself hardly significant, but as this rise is quite in accord with the behaviour of Bramley's Seedlings in the previous season, and with Newton Wonders in both seasons, it strengthens the evidence for a shift in the extinction point of N.R. to higher levels in the later phases of storage.

Blackman analysed a series of curves of CO_2 output by Bramley's Seedlings in gas mixtures containing different percentages of oxygen [Blackman, 1928] and inferred that such a shift occurs. Using the alcohol number as an index of N.R. we have verified Blackman's inference by our work on two varieties of apple.

(iv) *Varietal and seasonal differences as to the date at which the shifts of the extinction point begins.* We may first note that different varieties from the same orchard and picked at about the same time, and with a similar storage history, may behave differently in a given storage season. For example, graph II in Fig. 2*b* records but little zymasis in March 1932 in Bramley's Seedlings at concentrations of oxygen above 3 %. We conclude that the extinction point had just started to shift at this date. In striking contrast, the shift for Newton Wonders was well under way a month earlier. Indeed, for this variety, at this time, N.R. was not completely extinguished in quite high concentrations of oxygen. This may be because the senescent breakdown of the respiratory centres in the protoplasm of the cells had begun [see Fidler, 1933, 1]. This is possibly the inevitable fate of all apples kept in store, and it is conceivable that the first shift of the extinction point may be a sign of incipient disorganisation. Should this turn out to be the case, varietal differences in regard to this shift may prove to be related to the different keeping qualities of different varieties in a given season.

Secondly, variation has been observed in the behaviour towards oxygen of a given variety of apple picked from the same orchard in successive years at approximately the same time and stored and dealt with each year as nearly as was practicable in the same fashion. Fig. 2*b* clearly illustrates this seasonal difference for Bramley's Seedlings. A comparison of graphs I and II shows that the extinction point had shifted far more by February 1931 than by March in the following storage season. In fact this shift by March in the second season was no greater than that which had occurred by December in the previous season (see graph I, Fig. 1*b*). We infer that the date on which the seasonal shift in the extinction point begins is, for any given variety, determined by the impress made by the complex of significant environmental factors incident on the fruit whilst it is still on the tree. Kidd and West [1919–31] in their storage

trials have frequently reported that the impress of these factors persists in storage apples and governs their keeping qualities. There may be a relation between these qualities and the time at which the shift of the extinction point begins: and we found that Bramley's Seedlings kept better in 1931–2 than in 1930–1. The more rapid deterioration of this variety in the first season was possibly owing to the earlier shifting of the extinction point.

B. *The rates of CO_2 output and of carbon loss in relation to oxygen concentration.*

(i) *CO_2 output.* Blackman [1928] stated that the extinction point of N.R. is reached in that concentration of oxygen where CO_2 production is at a minimum, and he placed this minimum at 5 % oxygen. Our results accord well with his. Thus the graphs in Fig. 3 show that at all dates in 1931–2, as the oxygen concentration in the external atmosphere was increased, the rate of CO_2 output by Bramley's Seedlings first fell from the high value attained in nitrogen to a minimum value and then rose to the normal rate for air[1]. The extinction point again (see sub-section A) appears to be less than 5 % oxygen.

A few points are given for higher concentrations of oxygen. These suggest the continued upward drift reported by Blackman [1930].

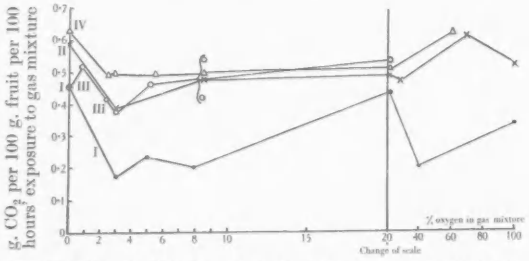

Fig. 3. CO_2 output by Bramley's Seedling apples during 1931–2. Corrected for conditions control values.

I, 2–18. ix. 31. ●
II, 11–18. xi. 31. ×
III, 6–13. i. 32. ○
IV, 16–31. iii. 32. △

Blackman concluded from the seasonal variation in the oxygen concentration at which the minimum CO_2 output was observed, that the extinction point for N.R. would move to higher oxygen concentrations as the fruit aged. Our conclusions in sub-section A support Blackman's views. Further, we note here that we have found for Newton Wonders in 1930–1 and 1931–2, and for Bramley's Seedlings in 1930–1, that the oxygen concentration in which the minimum value for CO_2 output was observed rose from 3–5 % oxygen to 8–10 % oxygen as the apples aged[2]. This rise affords further evidence that the behaviour of the apples we have used was substantially the same as that of the Bramley's Seedlings used by Blackman and Parija.

[1] The distribution of the CO_2 produced between N.R. and O.R. has been calculated, and the resulting values are given in Table II; but we have not collected enough data yet for gas mixtures containing less than 2·5 % oxygen to permit of critical analysis. We propose to perform further experiments in these low percentages of oxygen.

[2] The data on which this statement is based are included in Fidler's Ph.D. thesis [1932].

(ii) *Carbon loss.* In view of Blackman's calculations [1928] further data on the rate of carbon loss in relation to oxygen concentration may prove of interest.

We have obtained values for the rate of carbon loss under different conditions by simultaneously measuring CO_2 output and the alcohol number. We note that each carbon loss graph in Fig. 4 has the same form as the corresponding CO_2 output graph and has the same position for the minimum, *viz.* at about 3–5 % oxygen, the extinction point of N.R.[1] Carbon loss is greatest under anaerobic conditions. As the oxygen concentration passes from zero upwards, we observe that the first effect of oxygen is increasingly to restrict carbon loss. This restriction can be correlated with the gradual inhibition of zymasis as the oxygen concentration rises. Carbon loss appears to be at a minimum when zymasis has been completely inhibited. The second effect of oxygen, *viz.* to increase carbon loss by stimulating respiration, shows clearly after this minimum (see Blackman [1928]).

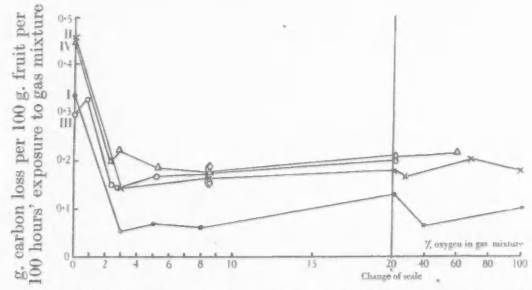

Fig. 4. Actual carbon loss by Bramley's Seedling apples during 1931–2.

I, 2–18. ix. 31. ●
II, 11–18. xi. 31. ×
III, 6–13. i. 32. ○
IV, 6–13. iii. 32. △

It will be noticed that the graphs I, II, III and IV lie at higher levels as the season advances. In all concentrations of oxygen a general seasonal stimulation of respiration probably contributes to this observed rise in carbon loss. In the lower concentrations of oxygen, moreover, the restricting effect of oxygen on zymasis may diminish.

Summary.

Apples have been exposed to mixtures of nitrogen and oxygen at 23° at different times during the developmental and storage life of the fruit. Using conditions controls rather than storage controls, and, allowing for experimental error and for sampling variation, we have found that:

(i) Rising oxygen concentrations progressively retard zymasis. The high rate attained in pure nitrogen falls until at a certain concentration of oxygen no alcohol is produced. This concentration is termed the extinction point of N.R. (nitrogen respiration).

(ii) Early in the storage season in Newton Wonder and Bramley's Seedling apples the extinction point lies between 1 and 3 % oxygen. There is some evidence that it may be near to 3 % oxygen.

[1] It was shown by Fidler [1932] that the same agreement holds between the extinction point of N.R. and the minimum carbon loss, early and late in the season, for (*a*) Bramley's Seedlings 1930–1, (*b*) Newton Wonders during three seasons.

(iii) The extinction point shifts to higher concentrations of oxygen later in the storage season.

(iv) In old apples alcohol may accumulate even in 100 % oxygen, *i.e.* there is no extinction point for N.R.

(v) The time of the beginning of the shift of the extinction point may be different for different varieties in the same season and for a single variety in different seasons.

(vi) The rates of CO_2 production and of carbon loss fall from maximum values in nitrogen to minimum values in 3–5 % oxygen early in the storage season, but in 8–10 % oxygen for older apples. The rates increase gradually when the oxygen concentration is progressively raised above these critical concentrations.

This research was performed under the auspices of the Department of Scientific and Industrial Research [see Fidler, 1933, 1]. Grants by the Royal Society and by the Research Committee, Armstrong College to one of us (M. T.) have helped on the research.

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CCXXIII. THE DIFFERENTIAL INACTIVATION OF INSULIN.

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Although one of the most characteristic effects of insulin administered to the normal animal is a reduction of the blood-sugar content it has also been amply demonstrated that the concentrations of inorganic phosphate [Wigglesworth *et al.*, 1922–23] and amino-acid-nitrogen [Luck *et al.*, 1928] in the blood are also reduced. These demonstrations have been made not only with the ordinary preparations of insulin used therapeutically but also with crystalline insulin.

The object of the experiments now to be discussed was to determine whether or not these three characteristics of insulin activity are functions of the entire molecule acting as a unit or of distinct portions of the molecule, acting more or less independently. In support of the unitary hypothesis is the common belief that phosphate-lowering, following insulin, is secondary to the hypoglycaemia, or at least intimately associated with the well-recognised changes in carbohydrate metabolism. Nevertheless, no satisfactory proof of such a relationship has ever been advanced. Nor is there any evidence that the effect of insulin upon the amino-nitrogen content of the blood is dependent upon the carbohydrate changes. It occurred to us that the possible existence of an interrelationship between these three physiological activities might be investigated directly through the use of partially inactivated preparations of the hormone.

It would be superfluous to discuss here in detail the extensive investigations of Freudenberg and associates, of Jensen, Scott, Du Vigneaud and others on insulin inactivation which have contributed much to our knowledge of the so-called "active groups" of insulin. In general, these investigations have been restricted to the chemistry of inactivation, employing as the sole criterion of activity the ability of the material to lower the blood-sugar content or, in large doses, to induce convulsions.

The present investigation is essentially an extension of such studies, the treated insulin being examined, not only in respect of its effect upon blood-sugar content but upon inorganic phosphate and amino-acid-nitrogen as well.

Experimental.

Inactivation of insulin. Two methods of inactivation were employed. In the first, crystalline insulin[1] was treated at 0° with 0·75*N* HCl in 75 % alcohol according to the method of Carr *et al.*, [1929]. 0·5 mg. samples were weighed

[1] We are much indebted to Dr D. A. Scott of the Insulin Laboratories, Toronto, and Prof. Du Vigneaud of George Washington University for the crystalline insulin used in this investigation.

out on a micro-balance and dissolved in 1 cc. portions of the acid-alcohol. The solutions so obtained were diluted to 12 cc. after 0, 1, 2, 3, 4, 5, 6, 7 or 8 days and thereupon injected at once into fasting rabbits.

In the second method, soft X-rays of high intensity were used as the inactivating agent. 0·2 to 0·5 cc. portions of insulin, 80 units per cc., were measured out into cylindrically ground depression slides, having a capacity of about 0·5 cc. The slides were sealed with thin mica cover slips (of known thickness) and vaseline and irradiated for periods ranging from 1 second to 2 hours.

The apparatus employed has been used by one of us (J. M. L.) in other experiments [Brown *et al.*, 1933] and was designed to give radiation of unusual intensity through a short target to specimen distance (3 cm.), silver anode, thin aluminium window (0·003 inch) and moderately high power (30 ma. at 50 kv.). The intensity as measured by an ionisation chamber approximated to 2500 Roentgen units per second[1].

Immediately after irradiation the specimens were diluted to 16–40 cc. (1 unit per cc. based on initial activity) and injected into fasting rabbits[2].

Experimental animals. Large female rabbits of 3–3·5 kg. weight, previously maintained upon a diet of barley and alfalfa were used. The animals were deprived of food for 2 days prior to injection.

Blood analysis. Blood samples, each of 5 cc. were collected, the first shortly before injection, the others at suitable intervals thereafter. In most of the experiments on inactivation by acid-alcohol only a single post-injection sample was drawn in order to minimise the chances of haemorrhage itself exerting an effect and thus leading to possible misinterpretation of results. In the rest of the acid-alcohol experiments, samples were drawn at 0, 1, 2, 3, 4, 5 and 6 hours. In all the X-ray experiments, the times of collection were 0, 1·5 and 3 hours.

Samples were analysed for reducing sugar, inorganic phosphate and amino-acid-nitrogen by the methods of Folin [1928], Fiske and Subbarow [1925] and Folin [1922] respectively.

Results.

In Table I we have presented the results of experiments on 85 rabbits, injected with preparations of crystalline insulin which had been treated at 0° with 0·75*N* HCl in 75 % ethyl alcohol. Since it is not feasible to present the individual results, we have averaged the values within groups and reduced the averages so obtained to a common base line. This facilitates comparison.

Percentage retention of activity has also been calculated according to Freudenberg's method [Freudenberg and Dirscherl, 1928] and the values incorporated in Table I. Reference to this portion of Table I permits the conclusion that after 2 or 3 days of treatment at 0° with acid-alcohol, 25–30 % of the amino-nitrogen-lowering activity is retained, 15–50 % of the sugar-lowering activity and 60–80 % of the phosphate-lowering activity. The ability of insulin to lower the inorganic phosphate content of blood seems to be most resistant to inactivation by this means.

To obtain more frequent samples 27 additional experiments were performed, in which blood-samples were collected at 0, 1, 2, 3, 4·5 and 6 hours after

[1] We wish to acknowledge, most gratefully, the aid of Mr Morden G. Brown in operating the X-ray equipment.

[2] Prompt use of the irradiated insulin is important. Long-irradiated specimens, permitted to stand overnight, were found to be completely inactive with respect to amino-acid-nitrogen, sugar and phosphate. It is possible that hydrogen peroxide formed on irradiation causes this secondary destruction.

Table I. *Inactivation of insulin with acid-alcohol at 0°.*

Time of inactivation (days)	Number of rabbits	Post-injection changes in blood composition. Three-hour post-injection values. Percentage of initial: Amino-N	Sugar	Phosphate	Percentage of initial activity retained*: Amino-N activity	Sugar-lowering activity	Phosphate activity
0	12	80	58	76	100	100	100
1	11	89	73	74	54	64	100
2	8	95	81	80	25	52	84
3	13	93	94	83	32	14	63
5	12	—	94	92	—	16	32
7	17	100	92	92	0	22	34
8	6	98	101	85	14	0	53
9	6	93	103	98	32	0	11
Control†	11	101	99	98	—	—	—

* Activity of untreated insulin regarded as 100. The following formula was used:

$$\text{Percentage of initial activity retained} = \frac{100\,(a-b)}{c},$$

where a = normal concentration of amino-N, phosphate, or reducing sugar in blood;
b = average concentration of given constituent 3 hours after injection with treated insulin;
c = average reduction in concentration of the given constituent 3 hours after injection with untreated insulin.

† Insulin not injected.

injection. The periods of treatment with acid-alcohol were 1, 3, 5 and 8 days. The results, expressed as concentrations of the various blood constituents after 0, 1, 2, 3, 4·5 and 6 hours, were averaged by groups and plotted.

In computing the percentage retentions of activity the area over each curve was calculated and the values obtained compared with the reduction given by untreated insulin. Corrections were made, necessarily, for the slight hypoamino-acidaemia and hypophosphataemia produced by bleeding (control experiments without insulin).

The results were qualitatively the same as those presented in Table I. After 5 days of treatment with acid-alcohol only 12 % of the sugar-lowering activity was retained, in contrast with 82 % of the phosphate activity.

In Table II the results of the experiments on inactivation with soft X-rays are collected. After 15 minutes of irradiation only about 25 % of the amino-acid lowering activity remained but this fraction persisted undiminished even

Table II. *Inactivation of insulin with soft X-rays.*

Time of inactivation (mins.)	Number of rabbits	Post-injection values after 1·5 and 3 hours. Percentage of initial: Amino-acids 1·5	Amino-acids 3	Sugar 1·5	Sugar 3	Phosphate 1·5	Phosphate 3	Percentage of initial activity retained: Amino-acid activity	Sugar activity	Phosphate activity
0	10	87	77	54	55	78	79	100	100	100
0·004–0·25	10	94	89	55	55	77	70	43	99	110
0·5 –4·0	6	88	87	58	60	74	69	75	91	118
15	8	93	93	79	91	86	87	29	37	60
30	6	98	94	81	95	88	87	20	31	55
60	6	96	94	104	107	92	88	29	−11	40
120	4	97	95	102	100	94	85	23	− 2·4	41
120*	2	96	86	106	120	91	79	45	−24	56
120†	2	104	97	111	121	93	86	9	−30	41

* 5 × dose. † 10 × dose.

after 2 hours' exposure. After 30–60 minutes of irradiation the hypoglycaemic activity of the insulin was lost completely. In fact, long-irradiated specimens tended to be hyperglycaemic—a property which became more evident when the irradiated insulin was injected in massive doses. As for phosphate-lowering activity, again we observed that this property of the hormone was the most resistant towards destruction. Even after 2 hours of irradiation, from 40 to 50 % of the initial activity remained. This lowering of phosphate, obtained in the absence of hypoglycaemia, demonstrates that a substantial portion of the activity of insulin towards phosphate cannot be secondary to the carbohydrate activity of the hormone.

Table II also contains the results of several experiments in which massive doses of the long-irradiated insulin (2 hours) were injected. The object here was to determine whether these larger doses might not increase, substantially, the changes in concentration in the blood of the substances studied.

Although the amino-acid changes were inconclusive and the decrease in phosphate activity remained at 50 or 60 %, the most interesting observation was the unmistakable tendency towards hyperglycaemia. Doubtless several explanations can be advanced to account for this phenomenon. However, we are disposed to think that the irradiated insulin induces an increased secretion of adrenaline. If so, the persistent though lessened activity towards amino-acids and phosphate may be due to adrenaline.

As an incidental observation, it should be mentioned that these massive doses of irradiated insulin failed to induce convulsions.

Discussion.

All the experiments described in this paper agree in showing that the phosphate-lowering activity of insulin is the most resistant to destruction by the methods of inactivation examined. They demonstrate that insulin may be differentially inactivated—in the sense that the physiological activities of insulin may be separately destroyed. A complete separation was not accomplished, inasmuch as the loss of hypoglycaemic activity was accompanied by a loss of 50 % or so of the phosphate-lowering activity. Nevertheless it is evident that these three activities of the hormone are not lost in unison as the unitary hypothesis[1] of insulin structure and function would demand. The results permit no final conclusion as to the relative ease of destruction of the amino-acid-lowering activity, although our experiments with brief periods of X-ray irradiation frequently indicated that this factor is less stable to X-rays than the carbohydrate-lowering factor.

Here it should be mentioned that controls have been run with untreated insulin administered in dosages of 1 to 1/16 unit per kg. in order to see the effect of mere insulin dilution upon the blood-constituents[1]. We had in mind the possibility that the "differential" inactivation here reported might be more apparent than real; that inactivating agents, instead of affecting one kind of active group to a greater extent than another, might inactivate completely a fraction of all the molecules present—leaving the others unaltered. Such an explanation would be demanded by the unitary hypothesis. We found, however, that reduction of the quantities of insulin administered caused the activities with respect to blood-sugar and inorganic phosphate to be "diluted-out" in

[1] By the "unitary hypothesis" we mean that theory of insulin structure and function which postulates that the various physiological activities of insulin are the property of a single active "centre" or "unit."

[2] The experiments with these lower dosages of insulin were made by Mr Walton Van Winkle.

unison. These results will be included in a later paper of this series and need not be discussed further at this time. There was no indication, however, that low doses of insulin (comparable with a mixture of active and inactive molecules resulting from inactivation) would lower blood-phosphate without causing hypoglycaemia.

With respect to the X-ray experiments it is pertinent to mention that earlier attempts at inactivation (sugar-lowering activity) with X-rays have given only negative results. Den Hoed *et al.*, [1929] irradiated specimens for $6\frac{3}{4}$ hours from a tube fed by a current of 180 kv. and 2 ma. Since the target-to-specimen distance was 24 cm. the intensity of the incident radiation must have been only about 1/64 as great as that employed in our experiments. We found that an irradiation time of 30 minutes was necessary to bring about an appreciable reduction of the sugar-lowering activity of insulin. Under the conditions of the experiments of den Hoed *et al.* an irradiation time of 32 hours would be required to produce a comparable effect with radiation of the same wave-length. It is apparent then that the negative results obtained in earlier experiments are due, in large part, to insufficient radiation [*cf.* Nitzescu, 1924].

Inactivation by ultra-violet radiation has been reported by several investigators [den Hoed *et al.*, 1929; Kuhn *et al.*, 1931; Freudenberg *et al.*, 1930; Ellis, 1925; Freudenberg and Eyer, 1932]. Maximum absorption and inactivation are obtained by radiation of wave-length 2750 Å. [Kuhn *et al.*, 1931; Freudenberg and Eyer, 1932]. This corresponds with the principal absorption band of tyrosine and cystine and suggests that either or both of these amino-acids are so-called "active centres." But since the absorption of X-rays is probably atomic rather than molecular, there is little likelihood of the same mechanism operating in our experiments. It would be unprofitable, therefore, without many more data, to attempt an explanation of the mode of X-ray inactivation.

SUMMARY.

1. The inactivation of insulin by acid-alcohol and by soft X-rays has been studied.

2. In both cases the phosphate-lowering activity of insulin was the most resistant to destruction.

3. Massive doses of insulin inactivated by X-rays produced hyperglycaemia; about 50 % of the phosphate-lowering activity was retained.

4. It is concluded that insulin owes its multiple activity to a number of "active groups," "centres," or "units" which vary in stability towards inactivating agents.

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CCXXIV. THE EFFECTS OF INSULIN AND ADRENALINE ON THE AMINO-ACID CONTENT OF BLOOD.

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(*Received July 19th, 1933.*)

In previous work it has been shown that insulin, injected into the normal animal, reduces the amino-nitrogen content of the blood and tissues [Luck *et al.*, 1928; Kiech and Luck, 1928], the latter even though hypoglycaemia be prevented by the simultaneous administration of glucose [Luck and Spaulding, 1928–29]. The rate of urea formation is also increased. More and more we have come to believe that these effects upon protein metabolism are independent of the effects upon carbohydrate metabolism. In the present paper we wish to adduce additional evidence which leads to this conclusion.

Experiments with crystalline insulin.

First of all it became necessary to determine for a certainty whether the amino-acid-lowering activity of insulin, hitherto studied upon therapeutic preparations only, was possessed by the crystalline hormone. We were aware of the possibility of the amino-acid activity being due to associated impurities, in much the same way that the transitory hyperglycaemic action of amorphous insulin is probably due to some principle other than insulin itself [Bürger and Kramer, 1930; Geiling and de Lawder, 1930]. Professor du Vigneaud was good enough to send us a specimen of crystalline insulin with which we sought an answer to the question.

Seven rabbits, previously starved for 48 hours, were injected subcutaneously with a freshly-prepared solution of crystalline insulin (1 cc. per kg. of body weight of a solution containing 1 unit per cc.). Two blood samples of 2 cc. were drawn from each—the first a few minutes before injection, the second $3\frac{1}{2}$ hours after[1]. Reducing sugar and amino-acid nitrogen were determined by the methods of Folin [1922; 1928; 1929]. The following values were obtained.

									Mean
Amino-acid-N mg. per 100 cc.	Before	8·2	8·2	8·6	8·1	9·0	9·1	8·9	8·6
	After	6·7	6·9	6·6	6·5	7·4	7·8	7·4	7·0
Sugar mg. per 100 cc.	Before	99	96	97	94	92	94	94	95
	After	59	38	44	47	59	58	58	52

[1] To avoid hypoamino-acidaemia due to haemorrhage only two blood samples were drawn. Five animals receiving injections of saline in place of insulin and bled at 0, $\frac{3}{4}$, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$ and $4\frac{1}{2}$ hours after injection showed decreases in blood-amino-nitrogen content of 1·3 mg./100 cc. (mean value). Seven other rabbits which were bled but twice (0 and $3\frac{1}{2}$ hours after injection) showed no decrease. In two insulinised rabbits with six bleedings decreases of 3·2 mg./100 cc. were observed. The effect was maximum after $3\frac{1}{2}$ hours. These results are sufficient to account for our adoption of the "two-sample" procedure.

In the next paper of this series many more experiments, some with a different preparation of crystalline insulin, will be reported. The results are identical with these. It is apparent that the hypoamino-acidaemia produced by ordinary insulin is a result of the hormone itself, not of impurities.

Prevention of hypoglycaemia by the simultaneous injection of adrenaline. It occurred to us that a simple method of preventing hypoglycaemia arising from insulin administrations would be by simultaneous injections of adrenaline. An opportunity would thereby be presented of studying the rôle of hypoglycaemia in insulin hypoamino-acidaemia.

In the first group of experiments rats were employed. Attention was given to the effect of balanced injections of insulin and adrenaline upon the amino-nitrogen content of liver and muscle. By way of introduction it should be mentioned that earlier experiments demonstrated to us that the lowering of the amino-nitrogen content of blood induced by insulin is accompanied by a simultaneous reduction in the amino-nitrogen content of liver and muscle. This is in harmony with the finding of Kiech and Luck [1928] that the amino-nitrogen content of the animal as a whole suffers reduction.

In the present experiments 72 animals, starved for 36–40 hours at 28°, were used. The optimum conditions for obtaining a balancing of insulin hypoglycaemia by means of adrenaline were first determined. Samples were collected from the tip of the tail at −15, 5, 30, 60, 90 and 120 minutes after the initial injection. By repeated trial it was found that satisfactory control of the hypoglycaemia was obtained by injecting simultaneously 5 units of insulin per kg. of body weight, and 0·38 mg. of adrenaline per kg. followed by a second dose of adrenaline 65 minutes later. (0·250 mg. per kg.).

Blood-sugar content (mg./100 cc.).

Animal	−15 min.	5 mins.	30 mins.	60 mins.	90 mins.	120 mins.
21	91	99	121	112	106	96
22	86	106	119	124	115	119
23	101	109	134	112	108	102
24	96	129	166	143	123	102
25	—	88	118	116	108	92

In three other animals from each of which only one sample was collected (2 hours after the initial injection) blood-sugar values of 94, 111 and 82 mg./100 cc. were obtained. Three other animals, paired with these three, were similarly treated except that 0·9 % saline was injected instead of the adrenaline. Values of 40, 43 and 39 mg./100 cc. were obtained.

Amino-nitrogen determinations on liver and muscle. The animals, injected both with insulin and adrenaline as described, were killed 2 hours after the first injection. The liver and thigh muscles were promptly excised, frozen in liquid air, powdered and analysed for amino-nitrogen [Luck, 1928]. The mean values presented in Table I were obtained.

Table I. *Amino-nitrogen content of liver and muscle.*

	Liver		Muscle	
	Number of animals	Amino-N content per 100 g. liver mg.	Number of animals	Amino-N content per 100 g. muscle mg.
Saline controls	23	47·7	24	58·8
Insulin	15	44·9	19	54·7
Adrenaline	11	44·6	10	54·9
Insulin *plus* adrenaline	15	43·3	18	55·9

It follows that the reductions obtained in the amino-nitrogen content of liver and muscle by injection of insulin are not appreciably lessened, if at all, by the simultaneous injection of adrenaline. In fact, adrenaline alone was found to lower the amino-nitrogen content—a point of some importance to which we shall presently return.

The rats used in the experiments just described were analysed in pairs, so selected that the members of any given pair were of the same sex, closely similar in age and weight, and, when possible, of the same litter. The one member of the pair received either saline or insulin, while the other received either adrenaline or insulin *plus* adrenaline. Analysis of the data from such "paired" experiments led to the same conclusion. Thus we observed in muscle that although the amino-nitrogen content of the groups of saline controls varied from 57·6 to 60·2, the values obtained from the experimental animals paired with these were uniformly less. For example, six rats which received insulin had an average amino-nitrogen content in muscle of 53·0. The saline controls paired with these averaged 57·6. The decrease induced by insulin was 4·6 mg./100 g. The corresponding values for nine adrenaline animals and their saline controls were 54·7 and 58·2; net decrease 3·5. For six animals receiving insulin *plus* adrenaline the corresponding values were 56·8, 60·2 and 3·4.

The changes in the basal level of amino-nitrogen content, reflected in the shifting saline-control values, seem to be partly seasonal in nature. They are of greater magnitude in liver but may be satisfactorily controlled by adoption of the paired-animal method.

To obtain confirmatory data we approached the problem afresh by studying the effect of balanced injections of insulin and adrenaline on the amino-nitrogen content of the blood of rabbits. The animals were bled but once, $3\frac{1}{2}$ hours after the initial injection. The most satisfactory control of the hypoglycaemia was obtained by injecting simultaneously 1 unit of insulin per kg. of body weight and 0·075 mg. of adrenaline per kg. followed by two injections of adrenaline (0·05 mg. per kg.) after 65 minutes and 2 hours respectively. In two animals,

Table II. *The effect of balanced injections of insulin and adrenaline on the sugar and amino-nitrogen content of blood.*

	Sugar mg. per 100 cc. blood		Amino-N mg. per 100 cc. blood	
Animal	0	$3\frac{1}{2}$ hours	0	$3\frac{1}{2}$ hours
1 A	105	88	9·4	7·8
2 A	100	105	9·3	7·6
1 B	112	118	7·1	6·7
2 B	106	115	9·6	7·3
3 B	108	83	8·3	8·2
5 B	121	129	8·8	8·7
6 B	100	149	8·8	8·1
7 B	110	114	8·9	8·2
5 B	115	118	8·8	7·5
7 B	88	119	8·1	6·5
2 C	114	82	10·0	7·3
1 C	119	91	9·3	7·0
3 B	121	110	7·8	6·9
6 B	119	149	8·4	6·6
7 B	105	118	8·5	6·6
3 C	118	108	10·2	8·2
4 C	122	128	9·3	6·6
5 C	100	95	8·3	6·5
	110	112	8·8	7·3

so injected, five blood-sugar determinations were made during 3½ hours following the initial injection. All the values fell between 99 and 118 mg./100 cc. Eighteen experiments were performed. Despite the absence of hypoglycaemia a definite fall in amino-nitrogen was observed, the decrease being 17 % as against a mean fall of 21 % resulting from insulin alone. The means for all experiments follow:

Amino-N mg. per 100 cc.	Before	8·8	Sugar mg. per 100 cc.	Before	110·2
	After	7·3		After	112·2

. The complete results are presented in Table II. They indicate that a persistent hypoamino-acidaemia is obtained even when hypoglycaemia has been prevented.

Experiments with adrenaline alone.

Although the literature contains two papers [Loeper *et al.*, 1926; Putschkow and Krassnow, 1928] on the amino-nitrogen content of the blood of adrenalectomised animals and other communications on the effect of adrenaline upon various aspects of protein metabolism, the results reported are markedly discordant. Since no one seems to have studied the effect of adrenaline upon the amino-nitrogen content of the blood of normal animals, we undertook the experiments summarised in Table III. Fasting rabbits again were used. It will be seen that adrenaline is fully as effective as insulin in lowering the amino-nitrogen content of blood.

Table III. *The effect of adrenaline on the amino-nitrogen content of blood.*

	Sugar mg. per 100 cc. blood		Amino-N mg. per 100 cc. blood	
Animal	0	3½ hours	0	3½ hours
1 A	116	238	10·1	8·5
2 A	114	263	9·8	7·5
3 B	104	256	8·6	7·7
2 B	102	250	9·3	7·2
5 B	111	298	9·2	7·8
7 B	110	282	9·6	7·5
6 B	—	254	9·0	7·0
8 B	111	200	8·6	6·8
	110	255	9·3	7·5

Experiments with posterior pituitary extracts and insulin.

The known antagonism between certain pituitary principles and insulin [Burn, 1923; Olmsted and Logan, 1923] led us to investigate the effect of posterior pituitary extracts upon insulin hypoamino-acidaemia.

In the first group of experiments extracts of Armour's powdered posterior pituitary were used. The extracts were prepared according to the U.S. Pharmacopoeia [1926] and were based on the acetic acid extraction method of Aldrich [1908]. In the remaining experiments the oxytocic and vasopressor preparations of Kamm *et al* [1928] known as pitocin and pitressin were used[1]. Fasting rabbits were employed throughout. The results, presented in Table IV, show that pitocin with insulin caused very substantial decreases in the amino-nitrogen content of blood, the decreases being somewhat greater than we generally observe with insulin alone. At the same time the hypoglycaemic action of the insulin was reduced, especially with the smaller dose of pitocin (12 units/kg.).

[1] We are indebted to Parke, Davis and Company for supplying the pitocin and pitressin used in this work.

Table IV. *The effect of posterior pituitary extracts and insulin on the sugar and amino-nitrogen contents of blood.*

Sugar mg. per 100 cc. blood				Amino-N mg. per 100 cc. blood				Preparation used in conjunction with insulin (1 unit/kg.)
0	1 hour	2½ hours	3½ hours	0	1 hour	2½ hours	3½ hours	
117	75	49	52	8·6	8·3	8·3	8·2	Acetic acid extract 1 cc./kg.
98	73	51	56	9·7	9·4	8·5	9·0	
106	92	70	71	9·3	—	7·2	6·9	Acetic acid extract 2 cc./kg.
115	67	61	69	7·8	8·1	6·7	6·7	
125	112	89	99	8·1	7·2	6·4	6·2	Pitocin 12 units/kg.
101	133	95	80	9·2	8·8	7·3	7·0	,, ,,
113	73	68	60	10·8	9·5	8·2	7·9	Pitocin 18 units/kg. (divided dose)
122	70	63	66	10·0	8·6	8·0	7·9	,, ,,
110	80	67	69	8·0	8·7	6·8	6·8	Pitressin 10 units/kg.
100	117	—	65	8·1	8·5	6·8	6·8	Pitressin 15 units/kg.
114	74	71	78	9·0	10·0	8·4	7·9	Pitressin 15 units/kg. (divided dose)
93	83	69	57	8·9	9·6	7·8	7·2	Pitressin 20 units/kg. (divided dose)
110	118	69	65	9·1	9·6	8·1	7·9	,, ,,
114	123	61	71	8·8	9·1	8·8	8·5	,, ,,

Pitressin given with insulin reduced somewhat the hypoglycaemic action of the latter with little if any effect upon its amino-nitrogen-lowering activity, with the curious exception, possibly, of an early but transitory increase in the amino-nitrogen content [*cf.* Bischoff and Long, 1931]. The anhydraemia usually caused by pitressin may account, partially, for this increase in amino-nitrogen. In one animal hypoamino-acidaemia was not observed.

The results with the acetic acid extracts are not easily interpreted. The larger dose of extract decreased somewhat the hypoglycaemic effect of insulin without altering its ability to lower amino-nitrogen. The smaller dose was without effect in lessening the hypoglycaemia but, oddly enough, was accompanied by but little hypoamino-acidaemia.

It should be mentioned that controls with insulin, pitressin and acetic acid extracts alone were run. Pitressin alone caused a transitory increase in amino-nitrogen similar to that obtained with insulin and pitressin together.

In harmony with the adrenaline experiments the results indicate that the amino-nitrogen-lowering activity of insulin is independent of its hypoglycaemic activity. However, the neutralisation of the hypoglycaemia by the pituitary preparations was complete in only two cases, so the results, though supporting the conclusions drawn from the adrenaline experiments, do not constitute a complete proof in themselves.

Incidentally the data on blood-sugar lend support to the conclusion of Magenta [1929] that pitocin is more active than pitressin as an antagonist to insulin.

Discussion.

The principal result of these studies to which we wish to draw attention is the independence of the hypoamino-acidaemia induced by insulin of the characteristic effect upon carbohydrate metabolism—hypoglycaemia. An explanation, we feel, may be found in the experiments with adrenaline alone which caused decreases in amino-nitrogen content fully as great as those obtained with insulin alone. The fact that injected insulin provokes an increased secretion of

adrenaline [Cannon *et al.*, 1924; Houssay *et al.*, 1924; Kugelmann, 1931; La Barre and Houssa, 1932] leads us to suggest, in the light of our present findings, that adrenaline rather than insulin may be responsible for the decreases in amino-nitrogen reported in this series of papers. Other results obtained by the use of partially inactive insulin [Davis *et al.*, 1933] and low dosages of active insulin [Luck and Van Winkle] strongly support this conclusion. If adrenaline be the agent responsible for hypoamino-acidaemia, it is to be expected, as we have shown, that the prevention of insulin hypoglycaemia by adrenaline would be without effect upon the amino-nitrogen reduction. We are unaware of any work which indicates whether insulin would continue to provoke an increased secretion of adrenaline when hypoglycaemia is completely prevented by the simultaneous administration of glucose. Should it not do so, the observations of Luck and Spaulding [1928–29] could not be reconciled with the hypothesis that adrenaline, to the exclusion of insulin, is responsible for the hypoamino-acidaemia reported in this series of experiments. In consequence, the possibility that amino-acid lowering may be a primary and independent function of both insulin and adrenaline cannot be ignored. We hope that other experiments, to be undertaken before long, may give a conclusive answer to this question.

SUMMARY.

1. Crystalline insulin is as effective as amorphous insulin in reducing the amino-nitrogen content of the blood of normal rabbits.
2. The amino-nitrogen contents of liver and muscle are reduced alike by insulin, adrenaline, or balanced injections of insulin and adrenaline together.
3. Though hypoglycaemia be prevented by the use of balanced injections of insulin and adrenaline, hypoamino-acidaemia is still observed.
4. Adrenaline alone is as effective as insulin in reducing the concentration of blood-amino-nitrogen.
5. Pitocin lessens the hypoglycaemic action of insulin and increases slightly the amino-acid-lowering activity.
6. Pitressin given with insulin causes a slight and transitory hyperamino-acidaemia followed by hypoamino-acidaemia.

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CCXXV. STUDIES ON BACTERIAL PHOSPHATASES.

I. THE DECOMPOSITION OF PHOSPHORIC ACID ESTERS BY *CLOSTRIDIUM ACETOBUTYLICUM* WEIZMANN.

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The conception of the phosphoric esterification of sugars as a necessary step in their metabolism in bacterial cells follows logically from considerations of carbohydrate breakdown in muscle and in yeast. But the experimental evidence in support of this conception in the case of bacteria is not very extensive, and information regarding the bacterial phosphatase systems which are responsible for synthesis and hydrolysis of phosphoric acid esters is very limited indeed. Virtanen and Tikka [1930] succeeded in isolating from a mixture containing, initially, glucose, inorganic phosphate and dried *Bacterium casei* ϵ two hexosephosphoric acid esters. One of these resembled the Robison ester, while the barium salt of the other had the formula $C_{12}H_{19}O_{16}PBa_2$. This appears to be the only reported case where the product of the phosphoric esterification of a sugar, as effected by bacteria, has been isolated. Virtanen had previously obtained some evidence of such a transformation with *Bacterium casei* ϵ [1924], *Streptococcus lactis* [1925, 1] and *Bacterium acidi propionici b* [1925, 2], but the ester was not isolated in any of these experiments. However it has been shown that several species of bacteria possess an enzyme system which is able to form methylglyoxal from hexosephosphates. Such species are *Bacillus delbrücki* [Neuberg and Kobel, 1929], *Bact. coli* [Fromageot, 1929], *Bact. lactis aerogene* [Neuberg and Scheuer, 1930], tubercle and timothy bacilli [Kuroya, 1931], *Cl. acetobutylicum* [Pett and Wynne, 1932, 1] and *Propionibacterium jensenii* [Pett and Wynne, 1932, 2]. These findings suggest that hexosephosphates play a significant part in the carbohydrate metabolism of these organisms.

Very little information is available in the literature with respect to the hydrolytic action of bacteria upon phosphoric esters. Manning [1927] has shown that *Bact. coli commune* ferments the sodium salts of both hexosediphosphoric acid and "hexosemonophosphoric" acid synthesised by yeast. Under either aerobic or anaerobic conditions decomposition proceeded with the formation of products normally found in glucose fermentations. The concentration of the diphosphate used was about 2·5 %. The fact that the ester is fermented by *Bact. coli* is not, in itself, evidence that a phosphatase is present, since it is conceivable that the glycolytic mechanism of the organism is able to ferment the glucose portion of the ester without preliminary hydrolysis. Recent observations of Boivin and Mesrobeau [1933], however, have led to the conclusion

that a phosphatase is actually present in *Bact. coli*. In the present work it is shown that *Cl. acetobutylicum* possesses an active phosphatase, although sodium hexosediphosphate even in low concentration is not fermented by the living cells when no other carbohydrate is present.

Experimental.

In order to test the ability of the living organisms to ferment directly sodium hexosediphosphate, sterile solutions containing 1·2 % of the ester, 0·6 % of peptone, traces of NaCl and $MgSO_4$, and shredded filter-paper were inoculated with portions of suspensions of washed and of unwashed organisms removed from an active glucose-peptone culture at the age of 20 hours. The test media, contained in tubes, were incubated both aerobically and anaerobically at 37° and any gases formed in non-evacuated tubes were collected over dilute H_2SO_4. Similar tubes containing 3 % glucose–0·6 % peptone were treated in the same manner. The glucose tubes all yielded normal volumes of mixed gases, but not even after 2 weeks' incubation did any tube containing hexosediphosphate evolve a trace of gas. In all cases, however, growth occurred, due to the presence of peptone. The results indicate that sodium hexosediphosphate in the relatively low concentration of 1·2 % is not fermented by the living organism in the manner in which glucose is fermented. No attempt was made to determine whether any hydrolysis of the ester took place. In view of Manning's results with *Bact. coli*, the fermentation of the same hexosediphosphate preparation by the latter organism was tested. Media similar to those described above were inoculated with *Bact. coli* prepared according to the method described by Manning. Small portions of a suspension of organisms added to sterile glucose-peptone and hexosediphosphate-peptone solutions caused vigorous fermentation in 24 hours. The sodium hexosediphosphate used in these experiments and in those which follow was prepared synthetically with the aid of zymin made from brewer's top yeast.

The hydrolysis of sodium hexosediphosphate by Cl. acetobutylicum *in active glucose fermentations.*

Into each of nine tubes containing shredded filter-paper were introduced 15 cc. of 5 % glucose–1·0 % peptone solution; the tubes were sterilised, and varying amounts of sterile sodium hexosediphosphate solution of known strength in addition to sufficient sterile water to make the volume 25 cc. were added. Each tube was inoculated with 0·5 cc. of an active maize culture of the organism and incubated for 5 days at 37·5°. The liberated gases were collected over dilute H_2SO_4 and the volumes, corrected to 20° and constant pressure, were measured at intervals during the fermentation and at the end. The final inorganic phosphate content of all tubes was estimated by the Briggs method, final reducing power by the method of Shaffer and Hartmann. Sodium hexosediphosphate allowed the fermentation to proceed only when present in low concentrations. When the degree of hydrolysis of the added ester was plotted against concentration of the ester as in Fig. 1 it was observed that greatest hydrolysis occurred when the ester was present in a concentration of approximately 0·05 %. But it is also apparent from Fig. 1 that with initial concentrations of the hexosediphosphate higher than about 0·2 % practically no additional inorganic phosphate was liberated. This may possibly be explained on the basis that the available enzyme was practically completely saturated by 0·2 % hexosediphosphate or it may be due to an inhibitory effect of the accumulated inorganic

phosphate. The added hexosediphosphate did not affect the rate of fermentation except in concentrations higher than 0·35 % where there was definite retardation.

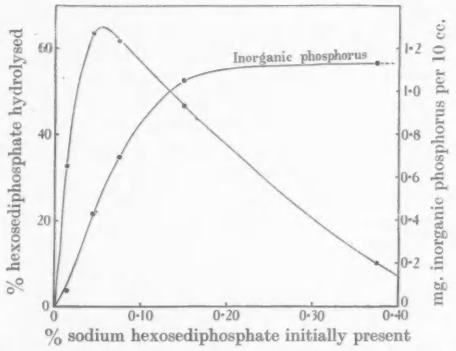
% hexosediphosphate hydrolysed
mg. inorganic phosphorus per 10 cc.
Inorganic phosphorus
% sodium hexosediphosphate initially present

Fig. 1. Hydrolysis of sodium hexosediphosphate in active cultures of *Cl. acetobutylicum*.

Since it was shown in the last experiment that added sodium hexosediphosphate was partially hydrolysed in active glucose fermentations, the rate of hydrolysis in relation to the general progress of the fermentation as indicated by the development of acid was next studied. For this purpose four flasks were prepared, containing the solutions specified.

(1) Experimental flask: 300 cc. of a sterile solution containing 3 % glucose, 0·6 % peptone and 0·04 % sodium hexosediphosphate; (2) three control flasks: (*a*) 100 cc. of a sterile 0·04 % solution of sodium hexosediphosphate; (*b*) 100 cc. of a sterile 0·04 % solution of the diphosphate in which were dissolved quantities of acetic and butyric acids such that their concentrations approximated to the amounts present in a normal 3 % glucose culture at maximum acidity; (*c*) 300 cc. of sterile 3 % glucose–0·6 % peptone solution.

Flasks 1 and 2 *c* were inoculated with 5 cc. of an active maize culture and all flasks were incubated at 37°. The changes in the total acid and inorganic phosphate contents of flask 1 are illustrated in Fig. 2. No significant change in

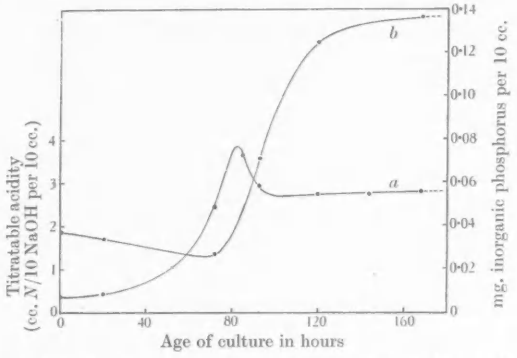

Fig. 2. Rate of hydrolysis of sodium hexosediphosphate in an active culture of *Cl. acetobutylicum*. *a*, titratable acidity; *b*, inorganic phosphorus.

inorganic phosphate occurred in any of the control flasks in 170 hours, but there was quite extensive hydrolysis of the hexosediphosphate in flask 1. The enzyme responsible for this hydrolysis did not come into effective action until the second phase of the fermentation. In this phase there is little or no further increase in the number of cells but frequently a decrease; during this period part of the accumulated acetic and butyric acids is converted into acetone and butyl alcohol at a rate which is comparable with the rate of accumulation of the acids in the first phase. The marked increase in the rate of hydrolysis in the second period suggests either that some of the cells have become more permeable to the enzyme or that the enzyme may have been liberated from cells which have actually undergone disintegration. It is not unlikely that a small portion of the liberated inorganic phosphate had its origin in the complex esters of the cell. The slight decrease in free phosphate in the early part of the fermentation suggests the formation of such esters during active growth.

Hydrolysis of glycerophosphate.

In the previous experiments the presence of a hexosephosphatase in active cultures was demonstrated: it was of interest to learn whether glycerophosphate, under similar conditions, would undergo hydrolysis. A series of flasks was prepared containing 3 % glucose–0·6 % peptone solution in which were dissolved varying amounts of sodium β-glycerophosphate (Boots); water was added to provide equal volumes in all flasks. The sterile media were inoculated with equal portions of an active maize culture of the organism and incubated at 37·5° for 5 days. From the amount of inorganic phosphate finally present in each flask the degree of hydrolysis of the glycerophosphate was calculated. The results are summarised in Table I. The amount of liberated phosphate increased with the concentration of glycerophosphate but the degree of hydrolysis calculated as % of added ester was low in all flasks, reaching a maximum when the concentration of the ester was approximately 0·02 %. Since glycerol is not fermented by this organism there can be no possibility that the accumulation of free phosphate is due to the operation of a glycerolytic mechanism which might conceivably release free phosphate by fermenting away the glycerol portion of the ester. It can be concluded, therefore, that a true phosphatase is present in the actively fermenting culture of the organism.

Table I. *Hydrolysis of sodium β-glycerophosphate in active cultures of* Cl. acetobutylicum.

Flask	Added glycero-phosphate %	Total inorganic P after 5 days mg. per 10 cc.	Inorganic P derived from ester mg. per 10 cc.	Ester hydrolysed %
1	0·00	0·086	—	—
2	0·00	0·087	—	—
3	0·015	0·130	0·044	20·45
4	0·030	0·172	0·086	20·00
5	0·075	0·177	0·091	8·46
6	0·300	0·343	0·257	5·97
7	0·350	0·407	0·321	6·40
8	0·500	0·330	0·244	3·40
9	0·750	No fermentation		—

Attempts to demonstrate synthesis of phosphoric esters.

Several attempts were made to demonstrate phosphoric esterification of glucose and fructose, in the presence of inorganic phosphate, by various preparations of the organisms. These included air-dried organisms, alcohol-ether-dried

organisms, dried organisms suspended in a small quantity of water and ground for several hours in an agate mortar with powdered glass and autolysed organisms. The results were all negative: in every case a definite increase rather than a decrease in inorganic phosphate was observed and, simultaneously, the reducing value of the mixture increased. Detailed consideration of these experiments is reserved until further studies have been made.

Summary.

1. Sodium hexosediphosphate in 1·2 % concentration was not fermented by living *Clostridium acetobutylicum*. When present in 0·75 % concentration the diphosphate completely inhibited the fermentation of glucose in an otherwise favourable medium. A similar concentration of sodium β-glycerophosphate inhibited the fermentation of glucose.

2. In active glucose-peptone cultures of the organism both sodium hexosediphosphate and sodium β-glycerophosphate were appreciably hydrolysed. The presence of a true phosphatase in active cultures has been established.

3. The hydrolysis of sodium hexosediphosphate proceeded most rapidly during the second phase of the fermentation following the time at which maximum acidity was attained.

4. Attempts to demonstrate the synthetic activity of the phosphatase failed.

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CCXXVI. STUDIES ON BACTERIAL PHOSPHATASES.

II. THE PHOSPHATASES OF *CLOSTRIDIUM ACETOBUTYLICUM* WEIZMANN AND *PROPIONIBACTERIUM JENSENII* VAN NIEL.

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Introduction.

In a previous paper [Heard and Wynne, 1933] the presence of a phosphatase in active glucose-peptone cultures of *Clostridium acetobutylicum* Weizmann was reported. It is the purpose of the present communication to describe some of the properties of this enzyme and of a similar enzyme present in *Propionibacterium jensenii* van Niel. Each of these organisms ferments various carbohydrates and certain related compounds. The first produces, as the chief end-products of glucose or starch breakdown, carbon dioxide, hydrogen, acetone, ethyl and butyl alcohols; the acetone and alcohols are derived from acetic and butyric acids formed as intermediary compounds. The second organism may be regarded as being representative of a rather large group of bacteria which ferment sugars and some related compounds with the formation of acetic and propionic acids [van Niel, 1928].

Phosphatases exhibit marked differences in their p_H-activity relationships. Those of mammalian tissues are, in most cases, much more active under alkaline than acid conditions, irrespective of the nature of the substrate. Usually the speed of hydrolysis reaches a maximum at about p_H 9, although, as Kay [1932] points out, this is probably only an apparent optimum since inactivation of the enzyme in more alkaline media may affect the exact identification of the optimum reaction. Roche [1931] observed that the phosphatase of certain mammalian erythrocytes appeared to differ from other mammalian phosphatases since it had a p_H optimum of 5·8 to 6·8 varying with the substrate. Among fungi and higher plants the few phosphatase systems which have been investigated are all most active under acid conditions. For example, the enzymes of *Aspergillus oryzae* and *A. niger* have p_H optima varying from 3·1 to 5·5 according to the method of preparation and the nature of the substrate [Asakawa, 1929]. Yeast phosphatase, whether acting hydrolytically [Kertesz, 1930] or synthetically [Euler and Nordlund, 1921], is most active at p_H 6·4, soya bean phosphatase at about p_H 5·3 [Kay and Lee, 1931] and rice phosphatase at p_H 4·3 [Horiuchi, 1931]. Regarding bacterial phosphatases very little is known. Virtanen [1927; Virtanen and Tikka, 1930] specified p_H 6·2 as the optimum for phosphoric esterification by *Bact. casei* ϵ. The fact that the phosphatase of *Cl. acetobutylicum* was found by Heard and Wynne [1933] to be active, hydrolytically, in growing cultures of the organism suggests that the enzyme prefers an acid medium. Quite recently it has been stated by Boivin and Mesrobeau [1933] that both *Bacterium coli*

and *Staphylococcus aureus* possess two phosphatase systems which exhibit optimum activity at p_H 7 and 10. This conclusion was based on studies involving the autolytic liberation of free phosphate from the complex esters of the cell. No systematic study of the phosphatase of any bacterial species has come to the attention of the authors.

Regarding the specific behaviour of the various phosphatases no conclusion can yet be reached. Strong evidence was advanced by Kay [1928; 1932] in favour of the identity of the phosphatases of mammalian bone, intestinal mucosa, kidney and blood-plasma. Recent observations, however [Roche, 1931; Jacobsen, 1931; Edlbacher and Kutscher, 1932] indicate that certain other mammalian phosphatases exhibit marked differences of behaviour. Moreover, the fact that phosphatases of higher plants and fungi have p_H optima which are widely divergent from the values characteristic of the mammalian enzymes may possibly be regarded as evidence of certain fundamental differences in the enzyme systems. The phosphatases of the two bacterial species investigated in the present work are shown to differ not only from mammalian and plant phosphatases but also from each other in several respects.

Experimental.

(A) *Materials and methods.*

Substrates. The glycerophosphates used were "sodium glycerophosphate 644" prepared by the Eastman Kodak Company and shown by Kay and Lee [1931] to be almost entirely the β-ester, and sodium α-glycerophosphate (Boots) which contained only small amounts of the β-ester. Unless otherwise stated, the term glycerophosphate, wherever employed in this paper, refers to the β-ester. Sodium hexosediphosphate was prepared from commercial calcium hexosephosphate somewhat after the manner described by Neuberg and Sabetay [1925]. The preparation, as finally used, still contained a small quantity of a monophosphoric ester and a trace of inorganic phosphate. The sodium pyrophosphate employed was Baker's C.P. grade and was heated to remove traces of orthophosphate. All these compounds give alkaline solutions; they were therefore adjusted to the desired p_H with HCl before using.

General experimental conditions. The reaction mixtures were usually buffered with 0·025 *M* potassium hydrogen phthalate, this concentration being an effective buffer but having no appreciable inhibitory effect on the enzyme activity. Toluene was added, and the mixtures were rocked in L-shaped tubes in a water-bath at 37·5°. At suitable intervals 2 cc. samples were removed and added to 10 cc. portions of 5 % trichloroacetic acid solution in centrifuge-tubes. After centrifuging, 10 cc. of the clear liquid were removed with a pipette, and the inorganic phosphorus was determined by the Briggs method. Control tubes containing substrate + buffer and enzyme + buffer were included in all experiments. Phosphorus values are expressed as mg. of phosphorus present as inorganic phosphate in 10 cc. of reaction mixture.

Preparation of the enzyme. Attempts were made in the case of each organism to determine whether phosphatase was present in the culture medium in which the bacteria had been grown. When the number of cells had reached a maximum they were removed in the Sharples centrifuge, the clear liquid was concentrated at low temperature to a twentieth of the original volume and was tested for enzyme activity. No phosphatase could be detected in any such preparation: the treatment was probably not destructive since amylase was always present in the concentrates of the liquid obtained from the *Clostridium* cultures.

It was found, however, that in the case of both organisms phosphatase can be shown to be present in the dried cells as well as in suspensions of living cells to which toluene is added. Dried preparations were found to be more stable and more convenient and therefore they were employed in preference to preparations of toluene-treated organisms.

Cl. acetobutylicum was grown at 37° in a medium containing 4 % glucose, 1 % peptone and shredded filter-paper. After about 20 hours' incubation the culture was filtered through cheese cloth and centrifuged. The organisms were washed twice with distilled water, spread on a glass plate, dried at room temperature in a current of air, ground to a powder and preserved in a desiccator. This dry material loses about one-third of its phosphatase activity in a month at 4° and becomes practically inactive after 5 months. Some variations were observed in the activity of the dried organisms depending on the age of the culture at the time of the removal of the cells. Greatest activity per g. of dried cells usually occurs at the age of 18 to 20 hours: this period in the fermentation of carbohydrate by *Cl. acetobutylicum* is a critical one, for it is at this time that the cell count reaches its maximum, the acidity reaches its peak, and the second phase of the fermentation begins. Just after the period of maximum acidity the phosphatase activity per g. of dried cells usually drops appreciably, but it then rises again to a value approaching the maximum, and, after several hours, it finally falls to a value which may be only a small fraction of the maximum activity. This final drop is frequently very rapid in cultures more than 60 hours old. The physiological significance of these variations is not clear. It is possible partially to remove the enzyme from the dried cells by simple aqueous extraction. From such extracts and others, more concentrated and considerably purified preparations of the enzyme can be made. In the present work, however, only the dried cells were employed.

Propionibacterium jensenii was grown at 30° for 7 to 10 days in a medium containing 3 % glucose, or 2 % glycerol, and 0·6 % peptone. Sterilised extract of fresh starch-free yeast constituted 10 % of the liquid of the medium; the remainder was distilled water. The medium was adjusted to p_H 7·1 before final sterilisation. After incubation the organisms were removed by centrifuging and were treated in the manner described above. The phosphatase of the dried powder is quite stable: in 6 months in the desiccator at room temperature about one-half of the activity disappears.

In the experiments reported the dried organisms were used in the proportion of 0·10 g. to 10 cc. of reaction mixture, unless otherwise stated.

(B) *Some properties of the enzymes.*

(1) p_H-*activity*. Each tube of a series received 5 cc. of phthalate buffer having approximately the desired p_H except in the alkaline range where no buffer was used, 0·10 g. dried bacteria and 5 cc. of 0·3 % substrate. The p_H of each mixture was determined electrometrically at the beginning and end of each digestion and if it changed by more than 0·2 unit the experiment was repeated. Under these conditions the rate of hydrolysis is constant for the first 90 minutes, but during the next half-hour the velocity begins to fall off quite appreciably. It is usually preferable in such experiments to use initial velocities as criteria of activity rather than the changes brought about during an arbitrary time interval or at equilibrium. In the present work, however, the same optimum p_H is obtained whether initial velocities or equilibrium values are plotted.

Results with Cl. acetobutylicum. With sodium glycerophosphate, either α- or β-, the optimum is at approximately p_H 5·1, the β-ester being hydrolysed more rapidly than the α-form. The optimum for sodium hexosediphosphate is at p_H 6·0. Fig. 1 illustrates the result of a typical experiment in which glycero-

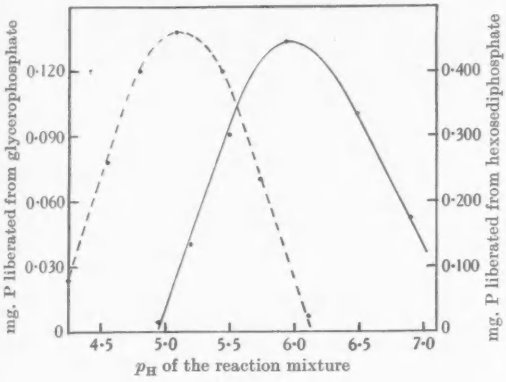

Fig. 1.

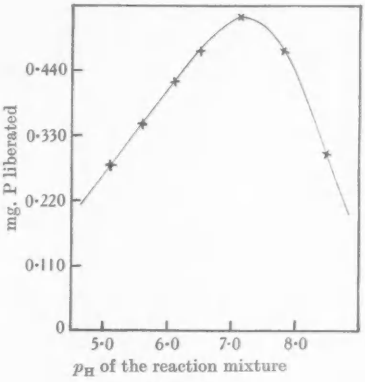

Fig. 2.

Fig. 1. The p_H-activity curves of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), and on hexosediphosphate.

Fig. 2. The p_H-activity curve of the pyrophosphatase of *Cl. acetobutylicum*.

phosphate and hexosediphosphate were hydrolysed. The organism also possesses a very active pyrophosphatase whose p_H-activity relationships are illustrated in Fig. 2. The optimum is at p_H 7·2.

Results with P. jensenii. With sodium glycerophosphate, either α- or β-, the optimum is at about p_H 7·0, the α-ester in this case being hydrolysed faster than the β-form. With sodium hexosediphosphate the optimum is at about p_H 6·0 and with sodium pyrophosphate at p_H 7·0. The pyrophosphatase is here

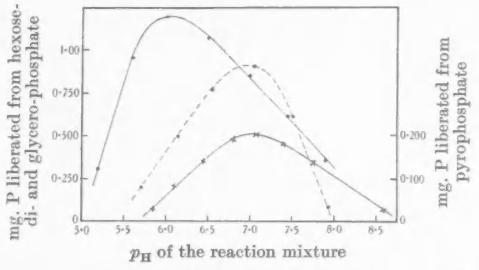

Fig. 3. The p_H-activity curves of the phosphatases of Jensenii acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

very much weaker than the other two phosphatases whereas in the *Clostridium* it is much more active. Fig. 3 shows the results of a typical experiment in which the propionic acid organism was used.

(2) *Relation of reaction velocity to enzyme concentration.* In establishing a correlation between enzyme concentration and speed of reaction it is necessary to choose a concentration of substrate such that the largest amount of enzyme present in any digest is completely saturated. Furthermore, since progressive inactivation of the enzyme frequently occurs in all but the very early stages of the reaction, consideration must be limited to the linear portions of the progress curves such as those illustrated in Fig. 4. By making use of such constant initial

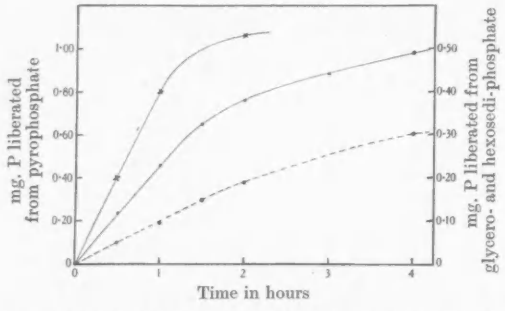

Fig. 4. Progress curves of the phosphatases of *Cl. acetobutylicum* acting on 0·15 % glycerophosphate (broken line), on 0·15 % hexosediphosphate, and on 0·5 % pyrophosphate (with crosses).

velocities it was found that, with the bacterial phosphatases, strict proportionality between speed of reaction and amount of enzyme was readily demonstrated. For this purpose a series of digests was prepared, each tube containing 5 cc. of buffer at the optimum p_H, 5 cc. of 1 % substrate, either glycerophosphate or hexosediphosphate, and increasing amounts of enzyme. In Fig. 5 is

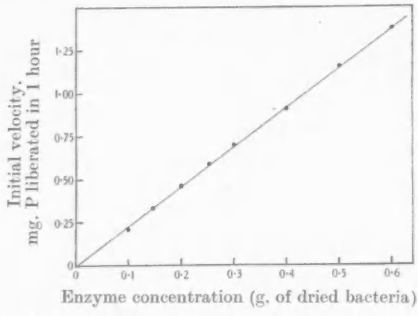

Fig. 5. The relation between enzyme concentration and rate of hydrolysis.

shown the result with the glycerophosphatase of the *Clostridium*, the speed of the reaction being expressed as mg. of phosphorus liberated as inorganic phosphate in 1 hour. The linearity is similar to that observed with other substrates and with the other enzyme preparation.

(3) *Effect of free phosphate.* Three solutions of NaH_2PO_4 adjusted to p_H 5·1 were prepared, of such strength that when 1 cc. portions of the solutions were diluted to 10 cc. the final concentrations of inorganic phosphate were those

listed in Table I. The inhibitory effects of these concentrations on phosphatase activity were determined in digests containing 5 cc. buffer, 0·1 g. dried organisms (*Clostridium*), 4 cc. of 0·4 % sodium glycerophosphate and 1 cc. of the phosphate solution. Control digests without added phosphate were run concurrently. The inhibitory effects, summarised in Table I, are of the same order of magnitude as those reported for mammalian phosphatase [Kay, 1928].

Table I. *The inhibition of phosphatase by inorganic phosphate.*

Initial concentration of phosphate	Degree of inhibition %
M/500	8
M/200	20
M/100	80

(4) *Effect of toluene.* Toluene was added in increasing amounts, up to 1 cc., to a series of tubes containing 0·1 g. dried organisms, 0·15 % substrate and suitable buffer in a total volume of 10 cc. Both organisms were used and both glycerophosphate and hexosediphosphate were employed as substrates but in none of the four experiments did the added toluene influence the rate or degree of the digestion. This result is at variance with the finding of Myrbäck and Euler [1929] who reported an inhibitory effect of toluene on the hexosephosphatase of dried yeast.

(5) *Effect of magnesium ions.* For the purpose of investigating the possible activating effect of magnesium ions the increase in inorganic phosphate in 1 hour was measured in a series of tubes each containing 0·1 g. dried organisms, 5 cc. buffer of the proper p_H, 4 cc. of substrate solution and 1 cc. of $MgCl_2$ solution of such strength as to give the desired final concentration. The concentrations of the three substrates, sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate, initially present in the digests, were 0·50, 0·18 and 0·50 % respectively. Suitable controls were run in all experi-

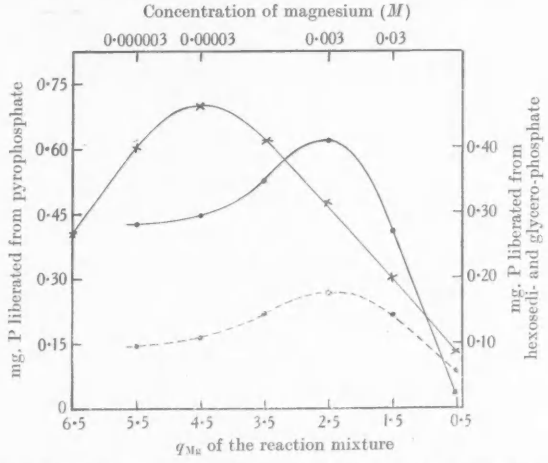

Fig. 6. The activity at different magnesium concentrations of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

ments. No difference could be detected between the action of $MgCl_2$ and $MgSO_4$. No significant change in p_H was observed in any digest during the period of the experiment. The range of magnesium concentrations investigated extended from 0·3 to 0·000003 *M*. Since such a wide range of concentrations is difficult to plot conveniently, the practice of Jenner and Kay [1931] has been adopted: the symbol q_{Mg} has been used to represent the negative logarithm of the molar concentration of magnesium. This symbol has a meaning similar to that of p_H but avoids the question as to whether the magnesium is present entirely in the ionic form.

In Fig. 6 the effects of different concentrations of magnesium on the phosphatase activity of dried *Clostridium* are illustrated. For the hydrolysis of glycerophosphate and hexosediphosphate the optimum concentration is q_{Mg} 2·5 and for that of pyrophosphate q_{Mg} 4·5. In each case it is observed that a sufficiently high concentration of magnesium retards the action of the enzyme.

With the propionic acid organism much greater activating effects of magnesium were observed. Typical results are illustrated in Fig. 7 from which it is seen that the optimum concentration is q_{Mg} 2·5 for the hydrolysis of all three substrates.

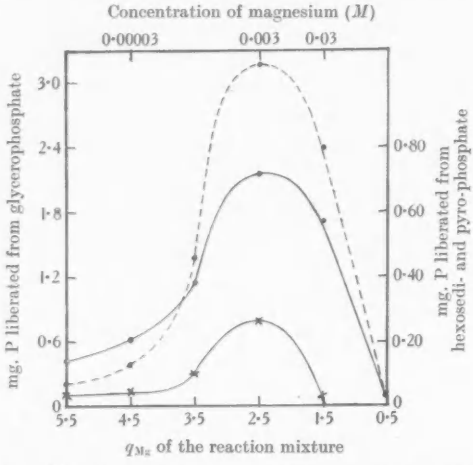

Fig. 7. The activity at different magnesium concentrations of the phosphatases of *P. jensenii* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

(6) *Effects of other ions.* In Table II are summarised the results of a study of the effects of several ions on the activity of the phosphatase systems of the two organisms. For purposes of comparison the value 100 is assigned to the activity in the absence of added ion and the relative activities as influenced by the various concentrations of ions are those listed in Table II. In the case of each substrate the same concentration was used throughout the series, namely 0·5 % sodium glycerophosphate, 0·18 % sodium hexosediphosphate and 0·5 % sodium pyrophosphate. The procedure adopted was the same as that described above for the study of the effect of magnesium, 1 hour's hydrolysis being chosen as a measure of activity except in the case of the hydrolysis of pyrophosphate by the propionic acid organism where 0·2 g. dried organisms was used and the

Table II. *Effect of various ions on phosphatase activity.*

Figures give the percentage hydrolysis under the influence of ions shown, as compared with the hydrolysis without added ions.

	Clostridium acetobutylicum									*Propionibacterium jensenii*								
	Glycerophosphate			Hexosediphosphate			Pyrophosphate			Glycerophosphate			Hexosediphosphate			Pyrophosphate		
Ion / Conc. of ion ...	*M*/10	*M*/333	*M*/2000	*M*/10	*M*/333	*M*/2000	*M*/10	*M*/333	*M*/2000	*M*/10	*M*/333	*M*/2000	*M*/10	*M*/333	*M*/2000	*M*/10	*M*/333	*M*/2000
Magnesium	88	175	150	51	148	125	82	150	200	500	1500	900	150	515	315	0	400	100
Beryllium	0	50	92	0	49	87	0	89	100	0	23	40	0	10	50	—	—	—
Calcium	77	89	95	98	99	100	50	100	99	30	55	90	25	60	90	20	70	94
Zinc	80	86	96	60	74	94	0	100	140	30	35	69	22	48	68	0	100	160
Strontium	46	56	100	34	81	94	0	90	103	20	30	90	18	29	80	—	—	—
Cadmium	50	62	100	42	82	99	0	85	100	30	60	97	22	48	85	—	—	—
Barium	40	90	95	60	79	89	0	66	95	98	99	96	90	98	101	—	—	—
Mercuric	0	35	40	0	26	60	0	30	39	0	45	50	0	0	22	—	—	—
Silver	0	50	80	0	10	50	1	59	95	0	0	35	0	0	50	—	—	—
Potassium	99	98	100	102	100	100	97	98	100	94	96	98	105	100	100	—	—	—
Sodium	100	100	101	100	99	99	95	97	100	99	99	100	98	99	100	99	98	100
Lead	0	90	100	0	100	100	52	72	96	0	40	80	0	30	60	—	—	—
Acetate	101	100	100	101	100	99	98	100	101	96	99	100	98	99	100	—	—	—
Nitrate	100	102	100	102	100	100	85	90	100	100	102	100	92	100	100	—	—	—
Sulphate	96	98	99	97	98	99	90	99	100	90	95	98	93	95	96	—	—	—
Chloride	99	100	100	100	100	101	95	99	100	101	100	99	100	99	100	97	99	99
	M/100	*M*/500	*M*/2000	*M*/100	*M*/500	*M*/2000	*M*/100	*M*/500	*M*/2000	*M*/100	*M*/500	*M*/2000	*M*/100	*M*/500	*M*/2000	*M*/100	*M*/500	*M*/2000
Cyanide	95	95	100	96	98	100	95	100	98	100	100	100	100	100	100	97	98	99
Fluoride	70	96	98	100	100	100	73	80	97	72	84	100	100	100	100	60	86	100

digestion was allowed to proceed for 4 hours. This reaction is much slower than the hydrolysis of the other two substrates by this organism. The figures in Table II are comparable for each substrate and are reproducible to within about 5 % with a given enzyme preparation. Portions of the same enzyme preparation of each organism were used throughout.

An examination of Table II shows that magnesium was the only ion which accelerated the rate of hydrolysis of all substrates by the phosphatases of both organisms. Zinc, in low concentrations, caused definite activation of the pyrophosphatase of both organisms. Fluoride, in certain concentrations, retarded the hydrolysis of glycerophosphate and pyrophosphate but not that of hexosediphosphate.

In conducting these experiments careful consideration was given to several important factors. The various solutions added to the digests were first adjusted to the p_H ultimately desired; the final p_H at the end of the experiment was determined in every case and, if a change greater than 0·3 unit occurred, the experiment was repeated. Secondly it was observed that in the presence of certain concentrations of some of the ions, particularly beryllium, barium, mercury, silver and lead, precipitation of the substrates occurred, a factor which may have led indirectly to retardation of the rate of hydrolysis. It is undoubtedly true also that the heavy metals have a direct inhibitory effect in certain concentrations. Finally it was necessary to consider the effect of each ion on the determination of inorganic phosphate from the points of view of the precipitation of the reagents and the development of the blue colour. Thus lead, silver and barium give such precipitates and were therefore removed with the proteins previous to the Briggs determination. Nitrate, cyanide and fluoride ions in 0·1 *M* concentration intensify the blue colour; a correction can be applied in the case of nitrates but not so accurately in the other cases. However, with cyanide and fluoride, no such disturbing influence was observed when the concentration was 0·01 *M* or lower.

Discussion.

Points of interest arising from the present work are those concerned with (1) the question as to the specificity of magnesium activation and the possible rôle of magnesium as a coenzyme of phosphatase systems, (2) the question as to the specificity of the phosphatases themselves.

Of all the ions investigated, zinc is the only one other than magnesium which exhibited any activating influence in the concentrations employed[1]. Zinc, it is true, appears to have a very limited, though quite definite, effect exerted only on the pyrophosphatase systems of both organisms; the degree of activation is of the same order of magnitude in the two cases and is confined to low concentrations of zinc. The limitation of the zinc effect to the pyrophosphatase in each case suggests that this enzyme differs in some manner from the hexosephosphatase and glycerophosphatase of the same organism. On the other hand the activation by zinc may be conditioned by the nature of the substrate rather than by that of the enzyme. The final interpretation of differences of this sort in relation to enzyme specificity must await further experimentation.

Magnesium, however, appears to stand apart from all other ions as an activator of all the phosphatase systems investigated in the present work. This result is in general accord with the findings of previous investigators who used

[1] Since these experiments were completed the arsenate ion has been found to cause a definite acceleration of the rate of hydrolysis of certain substrates by dried propionic acid organisms. An account of these observations will appear in a later paper.

mammalian phosphatases [Jenner and Kay, 1931]. The apparently specific response to magnesium of phosphatases in cells so different as those of mammalian tissues and bacteria, acting on various substrates, suggests that magnesium must play a fundamental rôle in the normal functional activity of these enzymes. It has been suggested that magnesium is a true coenzyme of the phosphatase system of mammalian tissues. In the case of the bacterial phosphatases studied in this work, this is rather unlikely: dried cells of *Cl. acetobutylicum* free from detectable magnesium were found to possess very appreciable phosphatase activity. For the purpose of testing this point the organisms were grown in a medium containing no magnesium other than that in the small amount of peptone present. 1 g. of the dried organisms was carefully ashed and the residue was examined for magnesium by a spot test, using *p*-nitrobenzoylazonaphthol [Feigl, 1931]. This test, sensitive to 0·0002 mg. of magnesium, gave negative results; the amount of magnesium present in 0·1 g. of the dried cells must indeed be negligible. In spite of this, when acting on glycerophosphate, 0·15 % in a total volume of 10 cc., the phosphatase present in 0·1 g. of the dried cells liberated from the ester 0·194 mg. of inorganic phosphorus in 2 hours. Obviously the enzyme did not require for its activity the presence of more than an infinitesimal amount of magnesium; it would therefore be incorrect to designate the magnesium ion as a coenzyme in the system under investigation.

Table III. *Comparative rates of hydrolysis of different substrates by bacterial phosphatases.*

Organism	Sodium glycerophosphate	Sodium hexosediphosphate	Sodium pyrophosphate
Clostridium acetobutylicum	1·0	2·0 ± 0·4	6·0 ± 0·6
Propionibacterium jensenii	4·5 ± 0·5	8·0 ± 0·2	0·4 ± 0·3

The experiments have also a bearing on the subject of the specificity of the phosphatases, though it is realised that a study of the enzymes of only two bacterial species does not justify any wide generalisation. Points of difference and of similarity in the respective systems of the two organisms are apparent. For example the propionic acid organism has a pyrophosphatase which is very much weaker than the other phosphatases, whereas with the other organism the reverse is true. This difference and some other relationships are illustrated in Table III. In this table are summarised data based upon experiments in all of which the concentrations of substrate employed were such as to make possible the complete saturation of the enzyme, the amount of the latter being constant throughout. It is apparent that the ratio of the rates of hydrolysis of the different substrates is not the same for the two enzyme preparations. There are other differences between the phosphatases of the two organisms. The p_H optima with sodium glycerophosphate as substrate are rather widely separated, p_H 7·0 for the enzyme of one organism and p_H 5·1 for that of the other. The propionic acid organism hydrolyses α-glycerophosphate more rapidly than the β-ester, whereas with the other organism the reverse is true. The phosphatases of the propionic acid organism are apparently much more extensively activated by magnesium than are those of the other organism, though this difference may be related to differences in the physical conditions of the crude enzyme-containing preparations which were used. In general however it can be said that the effects of the several ions on the activities of the enzymes are similar for the two organisms. The behaviour of the enzymes of the two species reveals other

similarities. The p_H optima for the hydrolysis of either hexosediphosphate or pyrophosphate are the same for the two organisms. Finally the peculiar effects of zinc and of fluoride ions are essentially the same for both bacteria.

From the point of view of the substrates themselves differences have been observed. Sodium fluoride in concentrations up to 0·01 *M* had no retarding effect on the hydrolysis of hexosediphosphate, whereas in considerably lower concentrations it retarded the rate of hydrolysis of glycerophosphate and of pyrophosphate. The differences in p_H optima were found to be sufficiently great in certain instances to suggest some form of specificity; it is possible that the use of purified enzyme preparations would reduce the differences, but it is unlikely that such optima as p_H 5·1 and 7·0 for the hydrolysis of different substrates could be shifted to the same level by methods of enzyme purification. Finally it may be noted that the optimum concentration of magnesium, namely q_{Mg} 4·5, for the hydrolysis of pyrophosphate by the propionic acid organism differs rather sharply from the optimum value, q_{Mg} 2·5, which characterises all other combinations of enzyme and substrate studied in the present work. This latter value is also optimum for the activity of mammalian phosphatases [Jenner and Kay, 1931].

Since the results reported reveal both similarities and differences a final decision with respect to the possible specific behaviour of the hydrolytic systems investigated cannot, as yet, be reached.

Summary.

1. The phosphatase systems of *Clostridium acetobutylicum* Weizmann and *Propionibacterium jensenii* van Niel have been investigated in a series of experiments in which sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate were employed as substrates.

2. Various properties and relationships of the enzymes have been established, including p_H optima, correlation between enzyme concentration and reaction velocity, the effects of free phosphate, of toluene and of magnesium and several other ions on the rate or extent of hydrolysis.

3. The significance of the observations has been discussed in relation to (*a*) the questions of the specificity of magnesium activation and of the coenzymic nature of magnesium ions and (*b*) the problem of the specificity of the enzymes.

The authors take this opportunity of expressing their gratitude to Dr H. D. Kay for helpful discussion of several problems and to Dr C. B. van Niel for a culture of the propionic acid organism. Generous contributions of calcium hexosediphosphate from the Winthrop Chemical Company, Inc., of Windsor, Ontario have greatly facilitated this work.

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CCXXVII. THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF ARSENIC.

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For the study of the effects of the arsenate ion on the activity of phosphatases an analytical procedure is necessary which will eliminate the usual interference of arsenate with the colorimetric determination of phosphate. The close chemical relationship of these ions and the need of avoiding hydrolysis of the residual phosphoric esters are factors which present serious difficulties in the solution of the problem in hand. None of the procedures described in the literature was found to be dependable, particularly with higher arsenate concentrations. A method has been evolved, therefore, which permits the accurate colorimetric determination of inorganic phosphate even in the presence of *M* arsenate.

Experimental.

Method of subtracting a correction for arsenate alone.

In the few reported studies of the effect of arsenate on phosphatases [Neuberg and Leibowitz, 1927; Mayer, 1928; Macfarlane, 1930; Lohmann, 1930; Braunstein, 1931; Morgulis and Pinto, 1932] the methods employed usually involved the use of a correction, though in some instances it is not clearly indicated how the arsenate effect was avoided. This correction is applied by subtracting from the arsenate-phosphate colour value, a value obtained for the same arsenate concentration alone.

Under the conditions in this laboratory the use of such a correction proved satisfactory only in the presence of small amounts of arsenate, and provided also that the time of colour development in the arsenate solution was, at the time of reading, precisely the same as with the solution containing arsenate and phosphate. With concentrations of arsenate higher than *M*/1000 this correction no longer gave reliable results, as shown in Table I.

Table I. *Showing the amount of "phosphorus" found in solutions of known arsenate and phosphate concentrations by subtracting a correction for the colour developed by arsenate alone. 0·1 mg. P was present in each case.*

Arsenate conc. in original solution	mg. P found after colour developing		
	5 mins.	60 mins.	120 mins.
M/10	0·460	0·296	0·171
M/100	0·264	0·200	0·115
M/250	0·181	0·150	0·108
M/500	0·138	0·108	0·104
M/750	0·121	0·109	0·103
M/1000	0·099	0·101	0·099
M/1250	0·101	0·102	0·101

The unreliability of the correction in higher arsenate concentrations appears even when using Lohmann and Jendrassik's modification [1926] of the Fiske and Subbarow method [1925] which, it has been claimed [Braunstein, 1931], gives dependable results owing to rapid development of the blue colour at 37°. The arsenomolybdate blue colour takes much longer to reach a stable intensity and to become proportional to the amount of arsenic present than is usually realised. Furthermore the mixture of arsenate and phosphate develops a blue colour at a rate even faster than phosphate alone. However, Table I shows that if the colours are permitted to reach a stable state, a condition which may require 2 hours or more, the correction can be more accurately applied. There remains, of course, the great disadvantage of having in some cases to apply a correction equivalent to 20 or more times the amount of phosphorus present.

Reduction of arsenate to arsenite.

At this point it was observed that arsenite, in contrast with arsenate, does not give a blue colour with phosphorus reagents, using either the method of Fiske and Subbarow [1925] or the Briggs [1922] modification of Bell and Doisy's method. This suggested reducing the arsenate to arsenite before determining the phosphate; details of a method for doing this have been elaborated.

Since this method was devised somewhat similar ideas have been found in two recent papers. Tananaeff and Potschinok [1932] suggested a procedure using Na_2SO_3 and HNO_3 before adding the regular reagents. A trial of the method as outlined, proved it to be satisfactory for amounts of arsenate up to about $M/500$, but not more. Tschopp and Tschopp [1932] in a valuable discussion of the formation of various "molybdenum-blue" compounds indicated that the addition of 1 cc. of 20 % $NaHSO_3$ just before adding the phosphorus reagents would prevent the formation of the arsenomolybdate. It is probable, however, that unless some time were allowed for reduction this procedure would not be very effective.

The reduction of larger amounts of arsenate requires appreciable time, but is accelerated by higher temperatures. Temperatures from 20° to 60° were tried, and 50° was selected as the most suitable for rapid reduction, having due regard to possible hydrolysis of residual phosphoric esters and to the removal of sulphur dioxide from the solution.

Effect of extra bisulphite on the phosphate determination.

The presence of sodium bisulphite in the usual phosphorus reagents had suggested its use for reducing the arsenate, rather than that of something which would have to be removed, such as H_2S; but it was necessary to determine the effects of extra bisulphite and of added acid on the accuracy of the determination. Table II shows the results obtained in the presence of varying amounts of sulphuric acid and of sodium bisulphite, using Fiske and Subbarow's method. Similar flasks were left at 50° for various times, as indicated, and the phosphate determinations were then made. Each 25 cc. volumetric flask had received 1 cc. of standard phosphate solution containing 0·1 mg. phosphorus, then the acid and the bisulphite; water was added to make a volume of about 10 cc. At the stated time the flasks were removed from the bath, the reagents were added, and the solution was diluted to the mark as usual. The conditions of the experiment permitted immediate reading of the colour intensity.

Comparing numbers 4 and 6 in Table II it is noted that an increase in the acid diminishes the recovery of phosphorus; a more striking effect is observed

Table II. *Showing the effect on the phosphate determination of varying amounts of 3N sulphuric acid and of sodium bisulphite, acting for different times at 50° before the addition of phosphorus reagents. 0·1 mg. P was present in each case.*

	cc. H_2SO_4	g. $NaHSO_3$	mg. P found		
			20 mins.	40 mins.	60 mins.
1	0	0	0·098	0·102	0·099
2	0·5	0·5	0·096	0·100	0·099
3	0·5	1·0	0·091	0·094	0·095
4	1·0	0·5	0·097	0·101	0·099
5	1·0	1·0	0·092	0·095	0·094
6	2·0	0·5	0·090	0·092	0·092
7	2·0	1·0	0·087	0·088	0·088

as the bisulphite is increased. It is therefore necessary to avoid large amounts of bisulphite. By calculation 0·1 g. of $NaHSO_3$ can produce enough SO_2 to reduce 20 cc. of *M*/10 arsenate. It was therefore decided that about 0·4 to 0·5 g. of bisulphite and 0·5 cc. of acid per flask would be safe to use and give a sufficient excess of SO_2. These quantities leave the bisulphite in excess because 0·5 cc. of 3*N* H_2SO_4 is equivalent to only 0·3 g. $NaHSO_3$; if, however, trichloroacetic acid is used to precipitate proteins it supplies the needed extra acidity. 1 cc. of 5 % trichloroacetic acid is equivalent to 0·03 g. $NaHSO_3$. Moreover, it is safe to increase the sulphuric acid to 1 cc. if desired.

The treatment with 0·5 cc. of 3*N* H_2SO_4 and 0·4 g. $NaHSO_3$ at 50° for 1 hour has no appreciable hydrolytic effect on 10 cc. of 2·5 % sodium β-glycerophosphate, and only very slight hydrolysis occurs with 10 cc. of 1·2 % sodium hexosediphosphate.

Table III shows the times necessary for the reduction of various concentrations of arsenate at 20° and at 50°, all in the presence of 0·1 mg. of phosphorus. When the recovered value approximates to 0·1 mg. of phosphorus, reduction is considered to be complete.

Table III. *Showing the colour, expressed as mg. of P, developed by 0·1 mg. P in the form of inorganic phosphate and varying amounts of arsenate after reduction had progressed for different periods of time, at 20° and at 50°. 0·1 mg. P was present in each case.*

Arsenate concentration in original solution	20°				50°			
	15 mins.	30 mins.	45 mins.	60 mins.	15 mins.	30 mins.	45 mins.	60 mins.
M/10	0·337	0·263	0·190	0·130	0·253	0·177	0·126	0·101
M/100	0·227	0·182	0·135	0·109	0·156	0·109	0·099	0·100
M/250	0·147	0·114	0·104	0·100	0·116	0·100	0·100	0·100
M/500	0·134	0·104	0·100	0·100	0·104	0·100	0·100	0·098
M/1000	0·112	0·100	0·101	0·099	0·098	0·099	0·100	0·098
M/2000	0·102	0·100	0·098	0·100	0·100	0·100	0·099	0·100
None	0·098	0·098	0·099	0·098	0·097	0·098	0·100	0·099

From Table III it will be noted that heating at 50° is distinctly advantageous, *M*/10 arsenate being reduced in 60 minutes; *M* arsenate was found to be completely reduced in 70 minutes. It is also noteworthy that the accuracy is not impaired by leaving the flasks at 50° for an hour, even though reduction may be complete in less time. The experiment shown in the table was performed with the 0·1 mg. phosphorus present in 2 cc. of the arsenate solution, but the

same result is obtained in about the same time if the phosphorus is present in 10 cc. of arsenate solution of the concentration indicated. Dilution up to 15 cc. does not noticeably affect the reduction.

Procedure.

The procedure used is, therefore, as follows. All or part of the trichloroacetic acid filtrate of the sample from an enzyme digest is transferred accurately to a 25 cc. volumetric flask as usual. To each flask are added 0·5 cc. of $3N$ H_2SO_4 and about 0·4 g. of $NaHSO_3$. The necessary bisulphite can be estimated with sufficient accuracy without weighing, after a little practice, and is introduced into the flask through a funnel. The flasks are then left in a water-bath at 50° (±1°), usually for one hour, after which they are removed, and the reagents are added for determining phosphate. The elevated temperature causes a more rapid development of the blue colour than usual, and readings may be made at once with Fiske and Subbarow's method, or in 15 minutes with Briggs's modification of Bell and Doisy's method. The standards should be developed for at least 3 minutes at 50° in order to ensure a stable colour intensity.

Using the procedure outlined, attempts were made to determine known quantities of inorganic phosphorus, each in the presence of different amounts of arsenate. Typical results are given in Table IV, where it is seen that the accuracy of the method falls within the usual limits of error for the determination of inorganic phosphate in the absence of arsenate or other interfering ions.

Table IV. *Showing the amounts of phosphorus found in mixtures of various amounts of arsenate and of phosphate, after reduction of the arsenate at 50°.*

	mg. P found				
	After 30 minutes' reduction of arsenate				After 60 minutes' reduction of arsenate
mg. P present	M/1000 As	M/500 As	M/250 As	M/100 As	M/10 As
0·050	0·049	0·048	0·048	0·050	0·050
0·200	0·199	0·198	0·197	0·200	0·201
0·300	0·296	0·296	0·300	0·300	0·306
0·400	0·400	0·400	0·400	0·400	0·396

With the aid of this method an investigation of the effects of arsenate on the activity of the phosphatases of bacteria, yeast, taka-diastase and mammalian tissue has been commenced.

SUMMARY.

In colorimetric phosphate determinations arsenate interferes by developing a blue colour with the ordinary reagents. In order to study the effects of arsenate on phosphatase activity this interference must be prevented, without, at the same time, hydrolysing the residual phosphoric esters. No method previously described was found to be sufficiently accurate in the presence of the higher concentrations of arsenate. A satisfactory procedure has been devised involving reduction of the arsenate to arsenite, a form of arsenic which does not interfere with the phosphate determination.

This study has been carried out under the supervision of Prof. A. M. Wynne, to whom the author expresses his indebtedness.

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CCXXVIII. STUDIES IN AVIAN CARBOHYDRATE METABOLISM.

IV. FACTORS INFLUENCING THE MAINTENANCE OF RESPIRATION IN SURVIVING BRAIN TISSUE OF THE NORMAL PIGEON.

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It is characteristic of the pigeon's brain that the rate of respiration falls very rapidly during survival in Ringer-phosphate solution. The addition of glucose [Gavrilescu and Peters, 1931] and more so of lactate [Gavrilescu *et al.*, 1932] increases the uptake over periods of an hour; even in this case the maintenance of respiration claimed by a series of workers for mammalian brain is not seen [*cf.* Loebel, 1925; Meyerhof and Lohman, 1926; Warburg *et al.*, 1924; Holmes, 1930; Quastel and Wheatley[2], 1932]. During the course of study of the catatorulin action of vitamin B_1 concentrates [Passmore *et al.*, 1933], we were obliged to attend to this matter. The problem was to determine the nature of the factors responsible for the gradual fall in respiration in phosphate buffer solutions. Though CO_2-bicarbonate gives different results with some tissues, the advantages of phosphate for control of p_H and rapid technique are great, and the differences from CO_2-bicarbonate may be less fundamental than they appear. The finding that there is a direct parallelism between the behaviour to vitamin B_1 in Ringer-phosphate of the minced brain and the functional state of the living brain shows that the behaviour under our biochemical conditions is not too artificial to be of value [Meiklejohn *et al.*, 1932].

Fig. 1 shows the behaviour of minced pigeon's brain, the hourly rate being plotted against the time. This shows changes better than the accepted method of plotting total respiration and time. It will be seen that none of the substrates induces maintenance. This is not a question of concentration of substrate; Gavrilescu *et al.* [1932] showed that K_m for *dl*-lactate was 0·024 *M*, making the concentration used here (0·033 *M*) practically maximum. The addition of galactose has only slight effect, though it was found by Sherif and Holmes [1930] to prolong respiration in the nerve (only after 4 hours).

This behaviour was interpreted as a gradual inactivation of some essential enzyme system, until the observations upon cyanide (Fig. 1, curve for succinate + KCN) showed that a respiratory system could remain in abeyance and then return to an original level[3]. Further study has shown that the addition of certain phosphorus compounds has a marked influence upon maintenance of respiration, when lactate is also present.

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[2] Quastel and Wheatley's [1932] curves fall off quite quickly (*e.g.* pig brain).

[3] This interesting recovery from cyanide deserves more study; the % inhibition noted is close to that of Dixon and Elliott [1929]. It probably indicates formation of aldehyde [*cf.* Wieland, 1932].

Lactate essential. The influence of lactate upon prolonging respiration of brain tissue as well as muscle tissue is well known, as is also the fact that the action of glucose is mainly due to the lactate formed from it. Fluoride, for instance, stops glucose respiration but not that of lactate [Holmes, 1930; Krebs, 1931]. Nevertheless lactic acid is still very generally regarded as a product of anaerobiosis, not essential *per se* in cell metabolism. The discovery that the "catatorulin" action of vitamin B_1 was generally maximum only when lactate was present supports strongly the hypothesis that this acid is essential for normal cell respiration.

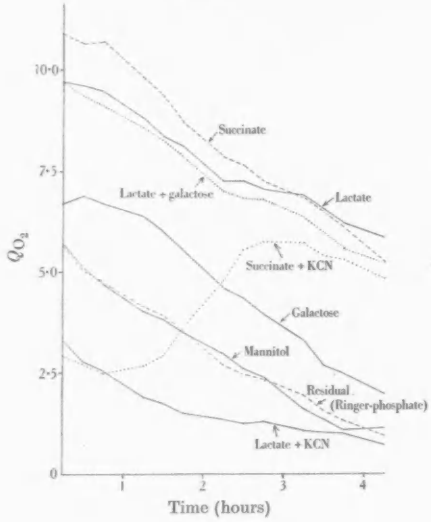

Fig. 1.

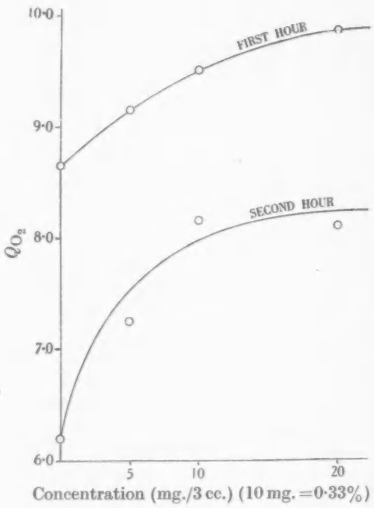

Fig. 2.

Fig. 1. Effect of various substrates upon respiration of minced cerebrum in Ringer-phosphate p_H 6·9. (Concentrations: lactate, 0·033 *M*; succinate, 0·011 *M*; galactose, 0·037 *M*; mannitol, 0·015 *M*; KCN 0·01 *M*.)

Fig. 2. Effect of concentration of candiolin upon respiration of minced brain in lactate, p_H 7·3.

It must be realised that no examination of a tissue has ever shown less than 10 mg./100 g. lactic acid; we may quote Davenport and Davenport [1928]; Fisher by use of iodoacetate in pigeon's tissues [1931]; Kinnersley and Peters in pigeon's brain [1930]; Owles [1930] found the minimum amount present in circulating blood to be 11 mg./100 cc. =0·0012 *M*; this may be concentrated at a surface to give 0·01 *M* in the tissue; comparison with the K_m curve referred to above, gives 0·024 *M* as concentration for maximum effect of the *dl*-acid.

If so, we can only reconstruct the normal respiration of the cell by adding to lactate other substrates. This kind of experiment has been done by others to study the "sparing" effect of one substrate upon another [Quastel and Wheatley, 1932], but not with the avowed purpose of using lactate as a basal "Ringer" metabolite.

It can be shown that addition of some substances to a basal Ringer containing lactate gives a much increased respiration, as compared with the respiration in presence of lactate or of the substrate alone. This was found to be the case with

crude Na hexosediphosphate (prepared from candiolin)[1]. The increase is more marked in the 2nd hour than in the 1st, and is maximum when 10–20 mg./3·0 cc. (0·03 *M* approx.) are added to the solution (see Fig. 2).

Such an addition effect is of two possible types; it may either (*a*) be due to independent respiration with two substrates; in this case the respiration of lactate *plus* the other substrate will not exceed the sum of the independent respirations with lactate and substrate separately; or (*b*) the increased respiration may occur only when the lactate and substrate are together; it may be a definite interaction. Exp. 1 (Table I) was designed to test this point.

Table I. *Exp. 1.*

O_2 uptake mm.³/g./hr. for successive half-hour periods. 1000 mm.³ = $Q_{O_2}5$.

Hours ...	½	1	1½	2	
Ringer-phosphate*	950	800	650	550	
Ringer + candiolin	1145	1015	845	710	About 200 mm.³ difference in each
Lactate only	1970	1750	1455	1275	
Lactate + Mg	1935	1750	1460	1280	No effect of magnesium
Lactate + pyruvate	2160	1920	1640	1500	
Lactate + candiolin	1995	1870	1660	1505	
Lactate + candiolin + pyruvate	1940	1820	1620	1480	
Lactate + candiolin + vitamin	1945	1840	1655	1505	

Conc. $MgCl_2$ 0·0016 *M*. Pyruvate 0·0038 *M*. Candiolin 0·33 %. Vitamin 1γ/3·0 cc.

* Calculated from a similar experiment.

The experiment is instructive; it shows increases by addition of pyruvate which equal that of candiolin; no increased effect with candiolin *plus* pyruvate, and as usual in the normal no change with vitamin addition[2]. But since candiolin increases respiration with the Ringer-phosphate solution alone, there is no evidence that the candiolin interacts only in presence of lactate; the effect is of type *a*. The feeling that there was some factor at work influenced by lactate led to the trial of hexosediphosphate. Exp. 2 (Table II) shows that hexosediphosphate as purified from commercial candiolin does not even give maintenance effect. (The action of fluoride will be discussed later.)

We believe that the factor especially concerned is pyrophosphate, to which we were led by experiment upon the "catatorulin" effect (see a further paper).

Table II. *Exp. 2.*

(Calculated hourly.) mm.³/g./hr.

	Hours ...	½	1	1½	2
	Ringer-phosphate	960	820	640	545
	R.P. + hexosediphosphate	1030	940	805	635
	Lactate	2140	1960	1735	1500
	Lactate + hexosediphosphate	1980	1900	1795	1525
Fluoride (0·008 *M*)	R.P. + fluoride	650	530	360	265
	R.P. + hexosediphosphate + fluoride	670	575	390	280
	Lactate + fluoride	1480	1270	950	625
	Lactate + hexosediphosphate + fluoride	1340	1200	920	630

Conc. of hexosediphosphate 0·008 *M*.

[1] The effect is much larger than that described by Quastel and Wheatley [1932] for the combination of succinate and lactate, though comparison is difficult as they worked with tissue previously autolysed for 3 hours.

[2] Sometimes vitamin addition shows a slight decrease in the normal.

Exp. 3 (partly shown in Fig. 3 and Table III) shows the effect especially well. It is supported by other experiments on the normal, which illustrate it in greater or less degree, and by the work upon the avitaminous brain. Here the action of pyrophosphate is additive only in presence of lactate[1].

Table III.

mm.³/g./hr. 1000 mm.³ = Q_{O_2} 5.

Hours ...	½	1	1½	2	2½	3
R.P.	1000	650	480	405	305	260
R.P. + pyrophosphate	810	530	470	380	320	350
L. + pyrophosphate + fluoride	1030*	(940)	530	490	390	310
L.	1960	1360*	1130	950*	790*	690*
L. + pyrophosphate	2070	1475	1390	1220	1210	1100
L. + pyrophosphate + Mg	2280	1860	1600	1510*	1430*	1280
L. + pyrophosphate + α-glycerophosphate	2655	2200	1900	(1880)	1710	1530
Same + Mg	2625	2100	2060*	1950*	1550	1400

* Bad duplicates. L. = Lactate.

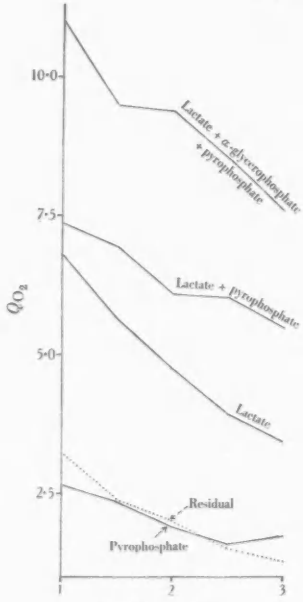

Fig. 3.

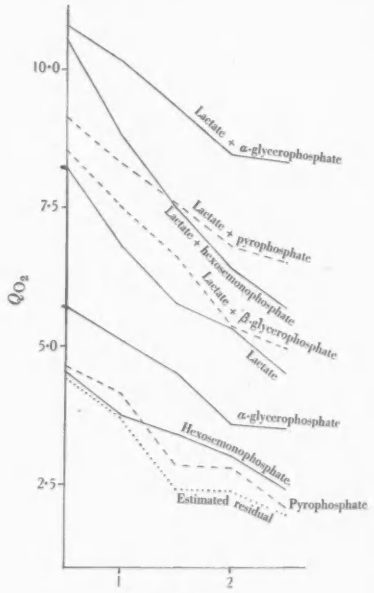

Fig. 4.

Fig. 3. Effect of pyrophosphate upon respiration of minced brain, p_H 7·3. (Concentrations: lactate, 0·033 *M*; pyrophosphate, 0·0093 *M*; α-glycerophosphate, 0·024 *M*.)

Fig. 4. Effect of various substrates upon respiration of minced brain p_H 7·3. (Concentrations: lactate, 0·033 *M*; α-glycerophosphate, 0·024 *M*; pyrophosphate, 0·019 *M*; hexosemonophosphate, 0·013 *M*; β-glycerophosphate, 0·023 *M*.)

[1] Controls with lactate + pyrophosphate and Ringer-phosphate solution with and without boiled brain show no oxygen uptake.

The additive action is an interaction of type *b*. So far this is the only case found and distinguishes the action of pyrophosphate from other active stimulants of respiration. It supports again the hypothesis that lactate is an essential component of the normal respiration system.

Exp. 4 (Fig. 4) shows the action of pyrophosphate; this and Exp. 5 (in Table V, also give the influence of α- and β-glycerophosphate and of Robison's hexosemonophosphate.

Davies and Quastel [1932] showed that bullock brain reduced methylene blue at about the same rates in presence of Na lactate and Na glycerophosphate, more slowly with succinate. Ashford and Holmes [1931] obtained twice the rate of respiration with lactate as compared with Na glycerophosphate.

We have found the β-glycerophosphate to be practically inactive. The effects obtained by others must have been due entirely to the α-glycerophosphate component of their preparations. This has a remarkable action, as can be seen by the abstract Table IV.

Table IV. *Exps. 4a and 4b.*

α-Glycerophosphate. (0·024 *M*) mm.³/g./hr.

	(4*a*)			(4*b*)		
Hours ...	1½	2	2½	1½	2	2½
Lactate only	1155	1070	900	1230	1140	890
α-Glycerophosphate only	900	720	705	920	850	610
L. + α-glycerophosphate	1810	1690	1665	1720	1620	1305
Ringer-phosphate only	—	—	—	475	400	330

Table V. *Exp. 5.*

mm.³/g./hr. 1000 mm.³ = Q_{O_2} 5·0.

Hours ...	½	1	1½	2	2½
R.P.	840	720	475	400	330
R.P. + α-glycerophosphate	1285	1100	920	850	610
R.P. + hexosemonophosphate	1140	900	650	550	440
L.	1955	1580	1230	1140	890
L. + α-glycerophosphate	2160	1980	1720	1620	1305
L. + hexosemonophosphate	2100	1670	1300	1160	900

The effect is largely independent of lactate, *i.e.*, it is of type *a*, and therefore differs from that of pyrophosphate in this respect. Exp. 5 shows that it has marked action apart from the lactate. As will be seen in a further paper, there is confirmation of this difference between the two substrates with the avitaminous brain, where vitamin interacts specifically with pyrophosphate but not with α-glycerophosphate.

Hexosemonophosphate promotes an increased oxygen uptake in the first hour, which soon drops either to the level of Ringer-phosphate or of lactate only, according to the constitution of the medium. It appears that there must be a rapid inactivation of the hexosephosphatase, but it is certain that this substrate (like hexosediphosphate) added to lactate alone induces no maintenance.

The finding that α-glycerophosphate is an independent additive substrate with lactate introduces at once the idea that we are dealing in brain with a system behaving like the new fermentation scheme of Embden *et al.* [1933], in which Meyerhof and McEachern [1933] concur. It is easy to understand that if free pyrophosphate is necessary in these experiments it may quickly diffuse from the cell and so rapidly fall to an ineffective concentration. The sudden

drop in metabolic rate would be then due to lowered concentration of essential substrates (lactate, pyrophosphate) for the energy metabolism, maintained by the cycle of carbohydrate change.

Exp. 6 (Fig. 5) and Exps. 7 and 8 in Table VI were designed to test whether a supply of several of the elements of Embden's fermentation scheme in presence of

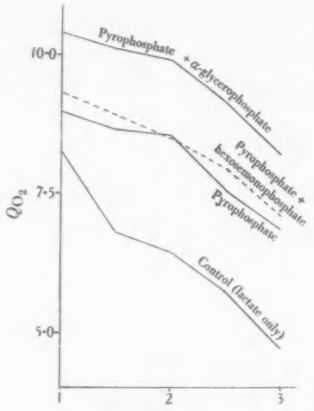

Fig. 5. Effect of combination of various substrates upon respiration of minced brain, in lactate, p_H 7·3. (Concentrations: lactate, 0·033 *M*; pyrophosphate, 0·0093 *M*; α-glycerophosphate, 0·024 *M*; hexosemonophosphate, 0·0072 *M*.)

Table VI. *Exp. 7. Combined effect of substrates.*

Hours ...	½	1	1½	2	2½	3
L.	1430	1100*	970	785	630	555
L. + α-glycerophosphate	2150	1450*	1130*	1070	1050	825
L. + hexosemonophosphate	1510	1220*	930	750	690	530
L. + pyrophosphate + α-glycerophosphate	2130	1900	1815	1410*	1290*	1140*
L. + pyrophosphate + α-glycerophosphate + fluoride	1555	1225	1010	900	750*	710
L. + pyrophosphate + hexosemonophosphate	1710	1430	1300	1160	1040	980
L. + pyrophosphate + α-glycerophosphate + hexosemonophosphate	2200	2000	1805	1700	1370	1400

Conc. α-glycerophosphate 0·024 *M*; hexosemonophosphate 0·013 *M*; pyrophosphate 0·0093 *M*; fluoride 0·008 *M*.

* Mg.

Exp. 8. Cerebrum only.

Hours ...	½	1	1½	2	2½	3	3½
L.	2500	1970	1605	1350	1080*	990	790
L. + pyrophosphate	2455	2170	1940	1830*	1480*	1850*	1435
L. + α-glycerophosphate	2700	2330	2110	1880	1250*	1780*	1510
L. + pyrophosphate + α-glycerophosphate	2625	2405	2170	1985	1670*	1800	1670
L. + pyrophosphate + α-glycerophosphate + hexosemonophosphate	2665	2400	2170	1970	1900	1775	1600

Conc. as in Ex. 7.

lactate would give sustained respiration in the "brei"; Exp. 6 (Fig. 5) shows that addition of pyrophosphate to lactate remarkably improves maintenance of respiration, and that α-glycerophosphate further increases respiratory rate. The addition of hexosemonophosphate sometimes has no effect (Exps. 6, 8), or a slight one in the first hour only. Probably there is inactivation of the phosphatase present. Though as yet not proved, it is hard to believe that these substances are not those originally present in the tissue which cause so high an initial level of respiration. There is still more to be learnt, as Exp. 8 shows; the respiration in cerebrum only is still not properly maintained; but for these three experiments we have as average at the 2nd hour lactate alone 1142 mm.^{3}/g./hr., + pyrophosphate and α-glycerophosphate 1791; and at 3rd hour lactate alone 828, with additions 1525, the remarkable average differences being 57 % and 84 % respectively.

Influence of fluoride. The presence of active fermentation following a scheme such as that of Embden and his colleagues in the respiring brain tissue can also be inferred from the effect of fluoride. This may be expected to influence any stage involving interaction with hexosephosphates. So, in Exp. 2, it is seen that some of the residual respiration is fluoride-sensitive and therefore due to hexosephosphate; this becomes some 5 % of the whole after 2 hours with added hexosephosphate. In presence of lactate, about one-third of the respiration is inhibited by fluoride; this is much more than even for Ringer-phosphate + hexosediphosphate. Hence, the improvement upon adding lactate is in part due to an increase in fluoride-sensitive respiration, the hexosephosphate (or triosephosphate) system.

In Exp. 7, fluoride reduced lactate + pyrophosphate + α-glycerophosphate to the level practically of lactate alone; all of this "extra respiration" is fluoride-sensitive. In Exp. 3, lactate + pyrophosphate is reduced to the level of Ringer-phosphate alone, again evidence of the interaction of the fluoride-sensitive system. The effect of α-glycerophosphate is to some extent independent of this: it is not fluoride-sensitive[1]. According to Meyerhof and McEachern [1933] fluoride stops either the breakdown of triosephosphate to α-glycerophosphate and phosphoglycerate, or the breakdown of phosphoglycerate. So oxygen rather than pyruvic acid appears to be acting as the hydrogen acceptor.

The above experiments have been done at p_H 7·3. Change of p_H to 6·6 causes 30 % or more fall in the respiratory rate, see Table VII.

Table VII. *Effect of* p_H *on respiration rate* (*mm.^{3}/g./hr.*), *0–1 hour.*

Exp.	p_H 7·3	6·6	Decrease (%)
9	1730	1390	−20
10	1990	1491 (4)	−25
11	1990	1080	−46

(4) = average of 4.

Magnesium. In all the experiments except Exps. 8 and 3, Mg was added in some form, to avoid any possible complications induced by lowered concentration in the action of the adenyltriphosphate coferment. In Exp. 3, tests with and without Mg do not show any consistent change. Since Lohmann [1931] found that the "lactic acid" coferment was not that of respiration, this is not surprising. In Exp. 1 $MgCl_2$ was added and in the others $\frac{1}{6}$–$\frac{1}{15}$ vol. of Ringer-lactate, saturated with magnesium phosphate.

[1] This has been confirmed by experiments on the avitaminous brain.

Discussion.

This work at present lacks the direct proof that the compounds added are the actual components of the tissue system; meanwhile, much may be done to help the study of this particular system by adding to Ringer-lactate pyrophosphate and α-glycerophosphate; 2–2½ hours of survival are so given. It seems unlikely that the effects are not general for brain tissue. The relation of pyrophosphate to active respiration may have a connection with the finding of Drury [1933] that pyrophosphate has stimulant effects upon the heart. For other tissues results may prove different. In phosphate buffers alone, Dixon and Elliott [1929] found that 0·033 *M* pyrophosphate inhibited markedly the respiration of muscle and liver tissue; there was a slight activation with 0·01 *M*. Engelhardt [1932] on the other hand inferred the participation of pyrophosphate from experiments with avian blood corpuscles showing the anaerobic splitting and aerobic synthesis of the easily hydrolysable phosphorus. Of the two paths of glycolytic change, one from glucose and one from glycogen, described by Ashford and Holmes [1929], it may be speculated that the precise function of that from glucose is to supply the essential component of the system, lactic acid; the other would then provide Embden's triosephosphates.

The case of pyrophosphate draws attention again to the necessity of bearing in mind possible concentration of diffusible constituents in the cell itself; this has been instanced recently by Jowett and Quastel [1933] for glyoxalase and glutathione. Comparison of tissue metabolism in different volumes may be dangerous near the critical margin for substances not strongly absorbed by the cell. A good quantitative illustration of this was given by Passmore *et al.* [1933] in the case of the critical level of residual tissue vitamin B_1. The optimum amount of pyrophosphate in the medium, 0·2 %, is of the same order as that possible in the cell, if we allow for some concentration at the active surface, from Lohmann's figures about 0·1 %.

Experimental.

Normal pigeons have been used throughout; they have been usually fed for 7–14 days upon the standard mixed laboratory diet. The technique of Passmore *et al.* [1933] has been used for the setting up of samples and obtaining oxygen uptake. 16 bottles can be filled from one mixed brain, making 8 duplicate estimations as a maximum. Values recorded are the mean of duplicates unless otherwise stated, calculated half-hourly.

Salts used. Na *dl*-lactate, made up in tubes from recrystallised zinc lactate and sterilised as before; 0·033 *M*.

Ringer-phosphate as before, but allowed to stand 2 days at p_H 7·3 before filtering.

Candiolin. "Ca hexosephosphate." Decomposed either by adding the required amount of Na oxalate, to the neutral suspension and allowing to stand, or by adding just sufficient HCl to dissolve and a solution of oxalate, and then immediately neutralising. The results did not differ. Tests showed that excess oxalate was not present.

Hexosediphosphate prepared as Ba salt from candiolin by Robison's method. Decomposed by Na sulphate.

Hexosemonophosphates. Specimen kindly supplied by Prof. R. Robison. Decomposed by Na_2SO_4 and standing some hours.

Ca α-glycerophosphate. Synthetic specimen supplied by Messrs Boots [King and Pyman, 1914]. Calcium removed by addition of oxalate; tests showed that excess oxalate was not present. Data supplied by Messrs Boots and Co. Loss at 140° 0·3 %. Residue on ignition 60·4 %. CaO 26·6 %. P_2O_5 34·0 %.

Na β-glycerophosphate. Pure salt, supplied commercially (Messrs Boots).

Na pyrophosphate. Prepared by igniting Na_2HPO_4 (Sørensen). This gave no phosphate reaction with molybdate unless it had stood.

The concentrations of salts are those giving approximately maximum effects.

Temperature 38° ±0·1. p_H 7·3, unless otherwise stated. For experiments at p_H 6·6, the Ringer-phosphate prepared at p_H 7·3 was treated with HCl.

In making additions to the Ringer-lactate solution, the salts have been made up in 0·2–0·3 cc. of solution and so added to the bottles, making allowance for this by addition of less Ringer-lactate. The changes in concentration of lactate so produced would not influence results, but this has been controlled in the latest experiments by adding the necessary amount for lactate in 2·0 cc. Ringer-phosphate and making up to 3·0 cc. with the various additions. The small variations in tonicity have not been corrected. It was found by one of us with Dr Gavrilescu that very wide variations in strength of a glucose solution had little influence upon oxygen uptake. This is not so surprising when it is remembered that in a recognised surgical method, hypertonic solutions are injected into the blood to produce shrinkage of the brain.

Summary.

1. The decrease in respiration rate in minced avian brain systems is substantially reduced in presence of lactate.

2. It is not reduced by galactose, mannitol, sodium β-glycerophosphate, hexosedi- and hexosemono-phosphates. The last two substrates may influence the rate of fall initially.

3. Na pyrophosphate added alone has little influence upon maintenance of respiration in the surviving tissue. Added with lactate, it much increases the respiration rate, values considerably in excess of those with lactate alone being obtained. Lactate and pyrophosphate are essential constituents of the respiration system.

4. α-Glycerophosphate has a remarkable additional influence. Ringer-phosphate to which lactate, pyrophosphate and α-glycerophosphate have been added gives much better maintenance than lactate alone.

5. Experiments with fluoride indicate that survival respiration in lactate-phosphate solution involves in part a hexosephosphate stage.

We are indebted to the Medical Research Council and the Christopher Welch Trustees for grants in aid of the work. Also to R. W. Wakelin for skilful assistance. We are further grateful to Prof. Robison for a specimen of hexose-monophosphate.

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CCXXIX. DISTRIBUTION OF SUGAR AND NITROGENOUS SUBSTANCES IN WHEAT GRAIN[1].

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The problem of the distribution of sugar and nitrogenous substances in wheat grain is of great practical and theoretical interest. A solution of this problem would provide the miller and baker with a number of indications concerning the composition of the different products of milled grain, enabling them to carry out the technological process upon more rational lines. The existing data on this subject are scanty.

Schulze and Frankfurt [1894] found that the germ of the wheat grain contains up to 25 % of sucrose on a dry weight basis. Apparently on the strength of this finding many subsequent investigators have considered that all the sugar of the wheat grain is concentrated in the germ. Having learned from a number of detailed analyses of wheat [Kiesel and Kretovitch, 1933] that in the whole ripe wheat grain the principal sugar consists of sucrose and that monosaccharides are almost absent, we have attempted to find out whether sucrose occurs in the endosperm as well as in the germ, and if so, whether the greater part of it is located in the outer or inner layers of the endosperm; also whether it can be found in the cells of the aleurone layer.

As regards nitrogenous substances we were interested principally in the question as to what portions are present in the cells of the aleurone layer, as it is known that flour from the latter fails to yield gluten.

We could not find in the literature data of direct analyses of the aleurone layer of wheat grain, although in the work of Teller and Teller [1932] on the nitrogenous substances of wheat bran it is indicated that the "epicarp" of the wheat grain contains little gliadin.

Experimental.

In order to determine the distribution of sugar between the germ and the other parts of the grain, a portion of the grain containing the germ was cut off from the rest and each part was analysed separately. As these analyses have shown (see below) that the portion of the grain without the germ[2] contained considerably more sucrose than the germ, it was desirable to obtain still more accurate determination of sugar distribution between the inner and outer layers of the endosperm and of the aleurone layer.

[1] The present investigation has been carried out in co-operation with S. S. Tanagos.

[2] Under the term "germ" we understand the portion of the grain called so by the millers, *viz.* the sprout.

For this purpose the grain was divided by a scalpel into three parts. First, the part containing the germ was cut off, and then the rest was divided into one part containing the inner kernel of the endosperm and another consisting of the bran coat with the aleurone layer and the outer layers of the endosperm.

Simultaneously with the analyses of the different parts of the grain an analysis of the whole grain was made as a check.

Material used for the investigation consisted of the grain "Cooperatorka" of the 1931 crop from the Crimea. The analyses were carried out according to the method of Kiesel [1930]; in the aqueous solution obtained after extraction of the finely ground material by 82 % alcohol and evaporation of the latter *in vacuo* and after clearing of the solution by lead acetate the following determinations were made: (1) *reducing sugars*, (2) *sucrose*—by the difference between the sugar determination obtained after 5 minutes' hydrolysis by 2 % HCl at 70° and (1), and (3) *resistant polysaccharides*—by the difference between the sugar determination after 24 hours' hydrolysis by 1 % HCl at 70° and (2). All determinations were made according to the Bertrand method and calculated on a dry weight basis.

A summary of the results of analysis of the grain divided into two parts—one containing the germ and the other without it—and of the control analysis of the whole grain is given in Table I.

Table I. *Sugar as % of dry weight of different portions of wheat grain.*

Material investigated ...	Part containing germ; 22·24 % of weight of grain	Part without germ; 77·76 % of weight of grain	Whole grain (control)
Reducing sugars	0·21	0·0	0·0
Sucrose	2·18	1·45	1·67
Resistant polysaccharides	0·0	0·0	0·0

By control computation of the data of the first two columns on the whole grain basis the following was obtained:

Reducing sugars	0·04 %
Sucrose 1·13 + 0·49 =	1·62 %
Total	1·66 %

Results of the investigation of the grain divided into three parts are given in Table II.

Table II. *Sugar as % of dry weight of different parts of wheat grain.*

Material investigated ...	A	B	
		Part without germ; 80·94 % of weight of grain	
	Part containing germ; 19·06 % of weight of grain	Outer layers of endosperm, with the bran coat and aleurone layer; 29·73 % of weight of "B"	Inner layers of endosperm; 70·27 % of weight of "B"
Reducing sugars	0·0	0·0	0·0
Sucrose	2·96	2·58	0·88
Resistant polysaccharides	0·01	0·0	0·0

Control computation of these figures on the whole grain basis shows the following ratio of the absolute quantities of sugar in the various parts of the

grain: 0·56 % (part containing germ) + 0·62 % (outer endosperm layer) + 0·50 (inner endosperm layer) = 1·68 %.

Material used for the investigation of the substances of the aleurone layer consisted of wheat grain, the germ and endosperm of which had been devoured by the storage weevil (*Calandra granaria* L.) by the ravages of which the inside of the grain had been cleaned out so neatly that the part left intact contained only the bran coat and the aleurone layer. Even under the microscope it was impossible to detect any particles of endosperm. Microscopic investigation showed that the cells of the aleurone layer and their contents had been left untouched by the weevil.

For the purpose of chemical investigation of the emptied grain it was subjected to a thorough grading and separation from foreign matter, ground as finely as possible and analysed for sugar content and various nitrogenous substances.

Repeated analyses have shown an absence of sugar from the empty grain and consequently from the aleurone layer.

Analyses of the nitrogenous substances contained in the empty grain, and consequently in the aleurone layer, have been carried out in the following manner.

A 10 g. air-dry aliquot of the material was extracted consecutively by water at 70–75° 4 times, 30 minutes each; by a 5 % solution of K_2SO_4 4 times, 3 hours each; by 70 % ethyl alcohol 4 times, 3 hours each; and lastly by 0·2 % solution of NaOH 4 times, 3 hours each.

Total nitrogen was determined by the Kjeldahl method in the original material, in the extracts obtained and in the residue.

Furthermore the aqueous extract was precipitated by normal lead acetate, and in the carefully washed precipitate a separate determination was made of the nitrogen of the water-soluble proteins; in the filtrate amide-N was determined by the Sachsse method and NH_2-nitrogen by the Longy method. Results of the analyses are summarised in Table III.

Table III.

Fraction of nitrogen	% of dry weight	% of total nitrogen
Total	3·54	—
Non-protein	0·88	24·85
NH_2	0·33	9·33
Amide	0·22	6·20
N of water-soluble proteins	0·47	13·28
N dissolving in 5 % solution of K_2SO_4	0·18	5·09
N dissolving in 70 % alcohol	0·21	5·85
N dissolving in 0·2 % solution of NaOH	0·96	27·10
N insoluble in 0·2 % NaOH	0·80	22·60

SUMMARY.

The data obtained permit the following conclusions.

Sucrose is contained not only in the germ of the wheat grain but also in the endosperm, the total amount being greater in the latter than in the former.

Considering that the weight of 100 whole grains is 3·200 g. and the weight of 100 empty ones 0·371 g., *i.e.* that the latter amounts to 11·6 % of the weight of the former, and taking into consideration the data of Table II, it is easy to estimate that the concentration of sugar in the outer layers of the endosperm is about 4·8 times greater than in the inner layers. This fact shows that the sugar is distributed in the grain in the same manner as is gluten [Cobb, 1925].

Analyses of the grain emptied by the weevil show that there is apparently no sugar in the aleurone layer.

As regards nitrogenous substances the results obtained by us confirm the data of Teller and Teller [1932], who found that wheat bran contains little gliadin. Our results indicate that there is practically no gliadin in the cells of the aleurone layer. Thus it becomes clear why flour from the cells of the aleurone layer does not yield gluten.

As regards glutenin and albumin, they compose the principal part of the protein substances of the aleurone layer, and the aleurone grains of wheat apparently contain a very insignificant amount of globulin. It is possible that during the process of extraction a part of the globulins may have passed into the water-soluble protein fraction, as the empty grains contain 6·57 % ash of dry weight substance, but the error caused by this factor could not be very great as in a volume of 100 cc. of the extracted liquid the concentration of salt was approximately 0·6–0·7 %.

Thus the general conception of aleurone grains as deposits of globulins does not apply to the aleurone grains of wheat.

In regard to the conclusions drawn from analysis of the empty grains it might be argued that the weevil while eating the grain might have caused some changes in the substances of the aleurone layer and that therefore the picture given by our analyses is not quite true. We do not however regard this as a serious objection since the microscopic appearance of the aleurone layer of such emptied grains is perfectly normal.

In conclusion we wish to express our thanks to Prof. A. R. Kiesel for his interest in this work.

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CCXXX. SOME CELL-WALL CONSTITUENTS OF *CETRARIA ISLANDICA* ("ICELAND MOSS").

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Chemical investigation of the lichen commonly known as "Iceland Moss" (*Cetraria islandica*) has extended over a considerable period of time, the earliest scientific examination of the material being recorded by Berzelius [1813]. In the main, most attention has been devoted to two groups of substances: (i) the so-called lichen acids and (ii) the water-soluble carbohydrate constituents (lichenin, *iso*lichenin *etc.*), which form the major part of the solids in the lichen. Concerning these latter a very large literature exists. The existence of a number of definite polysaccharides has been established and suggestions put forward for the constitution of certain of them. All appear to be derived from glucose, although Karrer and Joos [1924] reported the isolation of galactose and mannose from one of the fractions. The chemistry of these soluble carbohydrates has been studied so fully that in the present work no attempt has been made to investigate the subject further.

The lichen acids and the soluble polysaccharides comprise in all some 80 % of the total dry matter of the tissue. After their removal, there remains a residue, mainly carbohydrate in nature, consisting of cellulose, various hemicelluloses *etc.* It was this residue which formed the material for the present investigation. Hitherto it seems to have received little attention. Ulander and Tollens [1906] reported the presence of glucose, mannose and galactose therein, an observation confirmed by Hesse [1916]. Salkowski [1921], after hydrolysis of the water-extracted plant by dilute sulphuric acid obtained a resistant xylan-free ashless material, which he showed was not lignin. Poulsson [1906] reported the presence of 3 % of pentosan in the water-insoluble residue, basing his conclusion on a determination of furfuraldehyde. From the purely chemical standpoint, therefore, the present work was designed to furnish further information as to the chemical constituents of an already much-studied plant material. At the same time it was hoped that the results would make it possible to draw a comparison between the cell-wall components of this lichen and those of the higher plants, which have received so much attention in the past few years. The lichens differ so widely from the higher plants in their growth and habit, that it seemed of interest to discover how far the classes resembled one another in the chemical make-up of their cell-walls. It was found that, while there is a resemblance, in that cellulose and the hemicelluloses constitute a considerable proportion of the cell-wall of this particular lichen, the hemicelluloses are of a somewhat unusual type, apparently simpler in structure than those of the higher plants, in being free from pentose residues; while the pectins, of almost universal distribution in the cell-wall of the higher plants, are absent from this lichen. The possible significance of these differences is commented upon later.

Experimental.

1. *Preliminary examination of the lichen.*

In the first place a preliminary survey was made in order to estimate the relative amounts of fractions corresponding roughly to the different groups of carbohydrates present in the lichen. For each experiment, 100 g. of the oven-dried lichen, picked by hand free from extraneous material such as pine needles, were extracted with the following solvents, in the order given:

(*a*) Cold water: 12 extractions, each of 24 hours.

(*b*) Hot 95 % alcohol; 12 extractions, each of 6 hours.

(*c*) Hot water (90°–95°); 20 extractions, each of 6 hours.

(*d*) 4 % sodium hydroxide; 3 extractions at room temperature, each of 4 days, followed by 1 extraction at 80° for 6 hours.

At the end of this treatment, the residue was washed with dilute acetic acid until free from alkali, then with hot water and dried. The substances extracted were obtained in solid form, in the first three cases by direct evaporation on the water-bath, and in the case of fraction (*d*) (corresponding to the hemicelluloses) by precipitation with acetic acid and alcohol.

In the case of the hot water extractions, and to a less extent with the alkali, great difficulty was experienced in handling the material, on account of its extremely gelatinous nature, which made some mechanical loss unavoidable.

Estimations of furfuraldehyde and carbon dioxide yields were made on all the fractions, as well as on the original lichen. The carbon dioxide yields from all fractions were treated as if they arose entirely from uronic anhydride, except in the case of the alcohol extract, which contained the lichen acids—substances which might be expected to yield some amount of carbon dioxide on distillation with 12 % hydrochloric acid. The results of this preliminary examination of the lichen tissue are summarised in Table I.

Table I.

Fraction	Dry weight as % of lichen	Furfuraldehyde yield %	CO_2 %	Uronic anhydride %
Original lichen	—	1·48	1·65	—
Cold water extract	5·71	0·66	0·95	3·80
Alcohol extract	8·54	Trace	4·72	—
Hot water extract	64·20	1·29	0·85	3·40
NaOH extract	3·20	1·68	1·27	5·08
Residue	10·80	1·52	1·40	5·60

The results indicated that the fractions which it was proposed to study in detail (*i.e.* the "hemicellulose" and cell-residue) contained considerable amounts of furfuraldehyde- and carbon dioxide-yielding constituents, presumably of a "polyuronide" nature. The cell-residue, in fact, contained more of these substances than the hemicellulose fraction, indicating either a partial oxidation of cellulose units or the presence of substances of a "cellulosan" type resistant to extraction by the 4 % alkali usually employed.

It may be noted that the water-soluble fractions (lichenin and *iso*lichenin mainly) gave appreciable amounts of carbon dioxide, and thus appeared to contain uronic anhydride units, a fact which seems to have escaped notice hitherto. (It may be recalled, however, that Ulander and Tollens [1906] reported the presence of traces of pentosan in the lichenin fraction, based upon furfuraldehyde determination; since the uronic anhydrides themselves give

about 16 % of furfuraldehyde on distillation with HCl, it seems possible that the pentosan of Ulander and Tollens may have represented uronic anhydride units. The figures in Table I show that, in the cold water extract, the whole of the furfuraldehyde could be accounted for as derived from uronic anhydride; in the hot water extract about 0·5 % of furfuraldehyde cannot be so accounted for.)

2. *Attempted isolation of a pectin fraction.*

The preliminary analysis had shown that the amount of furfuraldehyde-yielding bodies in the lichen was small, none of the fractions giving so much as 2 % furfuraldehyde. Thus pectic substances were not expected to be present to any great extent; in view of their possible presence, however, several attempts were made to isolate a pectin fraction. Extractions were carried out on the water-extracted residue with hot 0·5 % ammonium oxalate and the extracts precipitated with alcohol. After redissolving in water and reprecipitating with alcohol the rather gelatinous material was again dissolved in water, and, in aliquot parts of the solution, pectin was estimated by the calcium pectate method. Only a very small amount of an acetic acid-insoluble calcium compound was obtained, and this bore no resemblance to calcium pectate as usually prepared. Further, the calcium content of the substance was found to be 16 % and its carbon dioxide yield 11·3 %, whereas calcium pectate contains 7·4 % Ca and gives 17·6 % CO_2. Two estimations gave a yield respectively of the calcium compound, 0·14 % and 0·42 % of the dry tissue after water extraction. Other experiments on similar lines failed to show the least trace of pectic substance, either in the tissue residue or in the water extracts, and the conclusion was therefore drawn that the lichen contained no pectic substances. The calcium compound obtained was probably the salt of one of the lichen acids.

3. *Extraction of hemicelluloses.*

The hemicellulose fraction was obtained by extraction of the cell-residue (after complete removal of the water- and alcohol-soluble material) with 4 % sodium hydroxide. The residue was treated, in portions of 200–300 g., with 4 litres of 4 % alkali, twice. The clear extracts, filtered through calico, were then made slightly acid with acetic acid and allowed to stand for some hours. Only in one case was a slight turbidity produced, but not enough solid separated to be removable by filtration or by centrifuging. A hemicellulose of the "A" type thus appeared to be absent.

To the faintly acid solution a half-volume of acetone was added, a copious precipitate being produced; after removal of this, the addition of excess acetone to the filtrate gave no further precipitate, so that the only hemicelluloses found in the sodium hydroxide extract were those of the "B" type [Norris and Preece, 1930]. This fraction was further separated by means of the copper method of Norris and Preece [1930] into fractions B1 and B2. Both were obtained in the form of almost white powders, of low ash content, quite stable in air and not darkening when heated to 100° for 4 hours. The relative yields obtained in different extractions varied somewhat, as shown in Table II.

Table II.

Extraction No.	Weight of lichen residue taken	Yield of hemicellulose	
		B 1	B 2
	g.	g.	g.
1 *a*	184	12·0	5·1
1 *b*	203	16·5	8·8
2	300	31·0	5·0

4. *Isolation of a further hemicellulose.*

It was noted, in the small-scale extractions, that a considerable amount of furfuraldehyde- and carbon dioxide-yielding material appeared to resist extraction with 4 % alkali (Table I). Examination of the corresponding residues after the large-scale preparation of hemicelluloses B1 and B2 confirmed this observation; further, the material on acid hydrolysis was found to give as much as 12 % mannose (estimated as the phenylhydrazone) and 5 % galactose (estimated as mucic acid). Evidently therefore polysaccharides other than cellulose were still present in this residue. Such resistant "cellulosans" are of course frequently met with in plant tissues, xylose being possibly the sugar most commonly concerned.

An attempt was made to isolate this more firmly combined hemicellulose by extraction with 17 % sodium hydroxide, the extraction being allowed to proceed for several days at room temperature. From the alkaline extract, no precipitate was obtained on acidification, but on subsequent addition of alcohol a bulky yellow-brown precipitate appeared. This was redissolved in 2 % sodium hydroxide and precipitated as a copper compound by addition of an equal volume of Fehling's solution; no further precipitation occurred on addition of acetone. The copper compound was decomposed with dilute acid and the hemicellulose obtained in the usual manner, the yield of the product varying from 7 to 11 % of the alkali-extracted residue taken (*i.e.* approximately 1 % of the original lichen).

5. *Examination of the individual hemicelluloses.*

Hemicellulose B1. The substance was soluble with difficulty in water, but its solutions were too strongly coloured to allow of a determination of optical rotation. The solution had no reducing action and gave no definite colour with iodine. It was easily hydrolysed to reducing sugars and gave a positive naphthoresorcinol test and a faint green colour with the orcinol reagent.

Determinations of furfuraldehyde and carbon dioxide in the usual manner gave: furfuraldehyde, 2·35 %, 2·32 %; carbon dioxide, 2·39 %, 2·47 %, equivalent to uronic anhydride, 9·56 %, 9·88 %. The furfuraldehyde yield was thus almost wholly accounted for by the uronic anhydride present, the balance corresponding to less than 1 % of pentosan. It was therefore concluded that pentose units were absent from this hemicellulose, which was composed of hexose and uronic anhydride units only.

A study of the rate of hydrolysis of the substance by dilute acid showed that hydrolysis was practically complete within 3 hours, using 3·5 % sulphuric acid (Table III).

Table III.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1·4 % sulphuric acid	3·5 % sulphuric acid
30	24·2	40·6
45	45·4	—
60	50·2	59·5
90	50·2	62·7
120	49·9	67·6
160	—	79·8
190	—	84·7
220	—	90·0
250	—	86·2

Accordingly, 2 g. of the hemicellulose were hydrolysed in this manner. During the hydrolysis, a small amount of an insoluble flocculum separated, and this was found to be unhydrolysed by 5 % sulphuric acid in 5 hours. (Some evidence was obtained that the amount of this product formed was dependent upon the conditions of drying the hemicellulose. Samples dried directly in the steam-oven, whereby the hemicellulose was obtained as a horny mass, gave as much as 17 % of the insoluble material, while those dried gradually through alcohol and ether gave only 6 % or less. Anderson [1931, 1, 2], working with hemicelluloses from cottonseed hulls, obtained 19 % of a "body X," apparently an impurity similar to this insoluble product; he considered it to be an essential part of the hemicellulose.) After hydrolysis, the greater part of the acid was removed by means of barium hydroxide; the filtrate was then warmed with excess of barium carbonate, filtered and the filtrate evaporated to a syrup *in vacuo*. The syrup was extracted several times with 85 % alcohol, and in the alcoholic solution the following sugars were identified.

Mannose: as phenylhydrazone. After removal of the phenylhydrazone, no glucosazone was formed on heating with excess of phenylhydrazine; glucose was therefore absent.

Galactose: as osazone, after removal of mannose; as mucic acid, by oxidation with dilute nitric acid.

Tests for other hexoses and pentoses were entirely negative.

The uronic acid present in the hydrolysate was isolated in the form of its barium salt by a method similar to that of Dickson *et al.* [1930]. From 10 g. hemicellulose B1, hydrolysed for 4 hours with a mixture of 12 g. sulphuric acid and 500 cc. water, 1·5 g. of a barium salt were obtained. This was found to contain 24·7 % Ba (barium uronate contains 26·1 % Ba). From this, a cinchonine salt of the uronic acid was prepared by the method of Nanji [1933], and had M.P. 172°, agreeing with that of a cinchonine salt prepared similarly from an authentic sample of galacturonic acid. On hydrolysis by the hydrobromic acid-bromine method of Heidelberger and Goebel [1927], crystals of mucic acid, M.P. 216°, were obtained, thus giving sufficient evidence of the presence of galacturonic acid, since galactose itself is not oxidised to mucic acid by the reagent used.

From these results it was concluded that the hemicellulose B1 of *Cetraria islandica* was a galactose-mannose-galacturonic acid complex. An estimation of the approximate amount of each of these units present in the hydrolysis mixture was made, mannose being estimated as its phenylhydrazone, and (mannose *plus* galactose) by copper reduction, after removal of uronic acid as the barium salt; the details of the methods used were those given in Browne's *Handbook of Sugar Analysis*. The average values obtained were: anhydromannose, 39·6 %; anhydrogalactose, 44·3 % (uronic anhydride, 9·74 %).

Partial hydrolysis of hemicellulose B1. It was noted during the study of the rate of hydrolysis of the hemicellulose by acids (Table III) that with 1·4 % sulphuric acid hydrolysis to the extent of about 50 % took place within 90 minutes, but that further treatment led to no increase in reducing sugars, but rather to a slight fall, due probably to the destruction of uronic acid [*cf.* Preece, 1931]. In order to study this apparent partial hydrolysis more fully, two portions, each of 3 g. of the hemicellulose, were hydrolysed with 2 cc. sulphuric acid and 250 cc. water for 90 minutes. The hydrolysate was in each case neutralised with barium hydroxide followed by barium carbonate, the precipitated barium sulphate with excess carbonate well washed with hot water, and the clear filtrates concentrated to 50 cc. *in vacuo* at low temperature. On

pouring the syrup into two volumes of alcohol, a brownish gum was thrown down, which remained without granulating when left in alcohol at 0° overnight. Two reprecipitations from aqueous solution by alcohol, however, sufficed to granulate the material, which was then dried by means of absolute alcohol and finally *in vacuo*. The amount of the product obtained was (*a*) 1·05 g.; (*b*) 1·51 g. From the alcoholic liquors of the second batch, 1·47 g. of sugars remained on evaporation.

(*a*) *Sugars.* In the alcohol-soluble fraction (sugars) only galactose could be identified. No trace of mannose phenylhydrazone was given under the usual conditions. It therefore appeared that galactose was the only sugar liberated by gentle hydrolysis.

(*b*) *Insoluble gum.* This fraction, when dry, was found to contain 5·8 % Ba—a figure far too low for a barium uronate and indicating that a fairly complex molecule still remained. After hydrolysis of the gum with 5 % sulphuric acid for 3 hours, mannose and galactose were identified in the hydrolysis mixture, and a quantitative determination showed that they were present roughly in the ratio 3 : 1. A carbon dioxide estimation on the original gum gave 8·6 % uronic anhydride (ash-free). The question of the structure of this gum, and of the parent hemicellulose, is discussed below.

Hemicellulose B2. This fraction resembled B1 in appearance and similarly gave a dark-coloured solution in water. It again had no reducing action but readily gave reducing sugars on gentle hydrolysis. Furfuraldehyde and carbon dioxide determinations gave the following results: furfuraldehyde, 1·72 %; carbon dioxide, 1·97 %, 2·06 %, equivalent to uronic anhydride, 7·88 %, 8·24 %. Again, the furfuraldehyde yield was almost completely accounted for by the uronic anhydride present, so that pentose groups were absent from this hemicellulose also.

The results of a study of the rate of hydrolysis by acids is shown in Table IV.

Table IV.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1·4 % sulphuric acid	3·5 % sulphuric acid
30	15·2	32·1
60	24·1	57·1
90	37·1	74·3
120	54·1	87·7
150	54·5	90·1
180	—	89·2
200	74·2	—
240	86·1	—
300	88·2	—

In the case of this substance, there was no evidence of any definite intermediate stage of hydrolysis by the weaker acid; on the whole, the substance seemed less resistant to acid hydrolysis than hemicellulose B1.

The sugars present were identified by the same methods as in the previous case, mannose and galactose being the only sugars found. The small amount of material available made impossible the isolation of the uronic acid as a cinchonine salt; however, a barium compound was obtained, which on oxidation with dilute nitric acid gave mucic acid, so that it was concluded that galacturonic acid was again present.

Estimations of the sugars present were carried out as described above, and the results are shown in Table V. There seemed to be a rather wider variation than usual between the amounts of the sugars found in two samples.

Table V.

Sample	Anhydromannose %	Anhydrogalactose %	Uronic anhydride %
1	21·1, 23·9	64·6	7·88
2	17·6, 17·6	60·1, 62·8	8·24

Hemicellulose extracted by 17 % alkali. This material, after purification by means of its copper compound, was obtained as a light-coloured powder, similar to the other hemicelluloses. It yielded 1·60 % of furfuraldehyde, and carbon dioxide corresponding to 10·7 % uronic anhydride; no pentose was therefore present. The sugars were again identified as mannose and galactose only, and the uronic acid as galacturonic acid by methods similar to those employed in the case of hemicellulose B1. Similar rough estimations of the proportions of the sugar units present gave: anhydromannose, 22·9 % anhydrogalactose, 62·9 %, figures very close to those obtained in the case of hemicellulose B2.

The course of acid hydrolysis of this substance was examined as before, the results being shown in Table VI.

Table VI.

Time of hydrolysis minutes	% reducing sugar liberated, calc. as glucose (Bertrand)	
	1·4 % sulphuric acid	3·5 % sulphuric acid
30	—	26·0
60	11·3	32·0
90	26·0	34·5
105	—	41·0
120	27·3	42·1
150	31·0	46·2
180	33·0	54·8
240	34·1	58·9
300	—	61·3

The substance was evidently much more resistant to acid hydrolysis than either of the hemicelluloses previously studied; it was apparently hydrolysed only to the extent of about one-third by the weaker acid, and again it was found that the only sugar set free during this partial hydrolysis was galactose. On further treatment of the portion unhydrolysed by 1·4 % acid within 4 hours, mannose and galactose were found in the hydrolysis-liquor in the proportion 1 : 2. In all cases examined, the uronic anhydride content of the products of partial hydrolysis was low (3–5 %), suggesting that very extensive decarboxylation had taken place under the influence of the acid, although the hexose units remained to some extent in combination.

Action of enzymes on the hemicelluloses. In view of the large amount of work recorded on the action of diastases on the water-soluble carbohydrates of *Cetraria*, a few experiments were made to test the action of these enzymes on the different hemicelluloses. Malt diastase was without action on any of the substances, but takadiastase produced about 30 % hydrolysis (measured by copper reduction) in 8 days in the case of hemicellulose B1; it was without action on the hemicellulose extracted by 17 % alkali. Attempts to isolate the products of the action of the enzyme failed.

6. *The cellulose residue.*

After very prolonged extraction with 17 % sodium hydroxide (lasting in all about 3 weeks), the residue was well washed with dilute acetic acid, until free from alkali, then with hot 50 % alcohol to remove sodium acetate and finally dried by means of alcohol, giving a dark brown fibrous material.

This substance swelled and dissolved slowly in Schweitzer's reagent and, with the exception of a very small dark residue, was soluble in zinc chloride-hydrochloric acid. On hydrolysis by the method of Monier-Williams [1921] it gave reducing sugars to the extent of 80 % (as glucose); from the hydrolysis mixture, a small amount (about 2 %) of mannose phenylhydrazone was obtained and large amounts of glucosazone, but no evidence of the presence of galactose could be found. It appeared that at least 80 % of this residue gave glucose on hydrolysis and could thus be regarded as cellulose.

A portion of the residue remained unattacked by the acid in the Monier-Williams hydrolysis, 0·4 g. of solid remaining from 5·1 g. of the residue. Similarly, on acetylation of 10 g. of the residue, 0·95 g. of substance remained insoluble in chloroform. It seemed therefore that about 10 % of the cellulose residue, *i.e.* about 0·8 % of the original dried lichen, consisted of this resistant, non-cellulosic material. It seemed possible that this substance was of lignin nature. Its dark colour precluded examination by any of the usual colour tests, and determinations of carbon and hydrogen gave figures intermediate between those required for cellulose and those accepted as an average for lignin, *viz.* C, 58·4 %; H, 6·1 %. A similar product obtained by Monier-Williams hydrolysis of a sample of lichen which had been extracted by water only (to avoid demethoxylation by alkali) was found to contain no methoxyl groups—always present in lignins. Thus the evidence was that the resistant material was not lignin. Finally, sections of the lichen failed to show the presence of lignin by the ordinary staining tests. There seemed no doubt therefore that the lichen contained no lignin, and the nature of the resistant material remained undetermined.

Discussion.

1. *Structure of the hemicelluloses.*

As shown above, approximately 90 % of hemicellulose B1 was accounted for as anhydromannose, anhydrogalactose and galacturonic anhydride, the ratio of the three units being approximately 8 : 10 : 2 (see however below). The nucleus stable to hydrolysis by 1·4 % acid was found to contain anhydromannose and anhydrogalactose in the ratio 3 : 1, with 8·6 % galacturonic anhydride. This product was apparently one of the complex acid substances which have often been isolated from hemicelluloses and gums [*cf.* Norman, 1929], although more complex than the aldobionic acids recognised as the nucleus of certain of the polyuronides. During mild hydrolysis of the hemicellulose, some two-thirds of the galactose was removed, none of the mannose and approximately half the uronic anhydride. That it was impossible to detect galacturonic acid in the hydrolysis mixture was not altogether surprising, in view of the rather unstable nature of these acids and the small amounts of substance used.

Combining the above figures, one may suggest that the original hemicellulose consisted of a resistant nucleus, containing 8 or 9 anhydromannose, 3 anhydrogalactose and 1 galacturonic anhydride units, together with a less resistant portion composed of 6 or 7 units of anhydrogalactose and 1 of galacturonic anhydride. More precise statements as to the proportions of the indi-

vidual units are not warranted, on account of the relatively approximate nature of the experimental methods involved.

A similar consideration of the figures obtained in the case of hemicellulose B2 suggested a molecule based on 9 units of anhydrogalactose, 3 of anhydromannose and 1 of galacturonic anhydride. In this case, however, there was no evidence of the presence of a stable nucleus in the molecule.

The "combined" hemicellulose again was found to be based on the same three units, in approximately the same proportion as in B2. Here, however, not only was the whole molecule rather more stable towards acids, but there was evidence of a more resistant nucleus, as in B1. Since, as shown in Table VI, the apparent end-point of hydrolysis depends on the strength of acid used, there is perhaps little justification for attempting to assign to the resistant nucleus a formula based on data given by a study of the action of acid of an arbitrarily chosen concentration. All that can be said is, that the hemicellulose contains a more resistant portion containing mannose, galactose and galacturonic anhydride, and a less resistant portion consisting of galactose and galacturonic anhydride only.

Three hemicelluloses have therefore been shown to be present in the cell-walls of *Cetraria islandica*, all based upon mannose, galactose and galacturonic acid in varying proportions. The dissimilarity between fractions B1 and B2 affords some evidence for the efficiency of the method of fractionation. It may be noted that, although these products were isolated by the methods used by Norris and Preece [1930], they differed widely in their chemical nature from the products obtained by these authors, which again supports the view, often expressed, that physical nature, rather than ultimate chemical constitution, determines the behaviour of a hemicellulose.

The most notable difference between these polyuronides and those generally obtained from the cell-walls of higher plants was the complete absence from the former of any pentose units. With the exception of certain hemicelluloses of seeds, which may contain hexose or hexose *plus* uronic acid units only, it seems that practically all the hemicelluloses so far isolated from widely different plants contain pentose groups in their molecule. The hemicelluloses of seeds are perhaps of definite "reserve carbohydrate" rather than "structural" nature; it may be that these lichen hemicelluloses combine the functions both of structural and reserve carbohydrate. It is at the moment impossible to amplify this suggestion; it would, however, be strange if the lichen, with its enormous reserves of glucose in the form of lichenin, should need to call upon the mannose and galactose of its hemicelluloses.

The only uronic acid detected was galacturonic acid, and, although mannose was present in quantities comparable with those of galactose, the corresponding uronic acid was not found. This fact, together with the observation that no mannose was liberated on mild hydrolysis, led to the suggestion that the mannose residues, after entering into combination, are in some way protected from oxidation (in the plant) and from hydrolysis (in the liberated hemicelluloses). It may be noted that, although the greater part of the total carbohydrate of *Cetraria islandica* consists of glucosans (lichenin *etc.*), no glycuronic acid has been detected, unless it be that the small amount of carbon dioxide liberated from the lichenin fraction was derived from this acid. One may suggest either that the glucose molecule when present in glucosans is rather less readily oxidised than that of galactose—the apparent non-existence of a highly oxidised glucosan corresponding to pectin is interesting in this connection—or else that glycuronic anhydride complexes pass into pentosans more readily than those of

galacturonic anhydride. This second suggestion certainly does not seem to apply in the present case, since pentosans are virtually absent from *Cetraria islandica*.

2. *The cell-wall of* Cetraria islandica.

The work here described, and that previously published, has established the presence of four main well-defined groups of carbohydrate in the cell-wall of *Cetraria islandica*, *viz.* lichenin, *iso*lichenin, hemicelluloses and cellulose; and the absence of pectins, pentosans and lignin. Of the carbohydrates present, lichenin, *iso*lichenin and cellulose represent condensation products containing glucose only as the parent sugar. The hemicelluloses (using the term in the general sense, to indicate substances resistant to water-extraction, but extracted from the tissue by dilute alkali), on the other hand, are based on mannose and galactose, with partial oxidation of the latter to its uronic acid; glucose is absent. Considerable differences exist, therefore, between the cell-wall constituents of this lichen and those of a typical higher plant. These differences must be accounted for by a different metabolism governing the formation of the cell-wall. The lichens present difficulties, which do not arise in the case of the higher plants, on account of their symbiotic nature; two distinct types of cell-wall, between which considerable differences of nature might be expected to exist, are therefore present. According to Escombe [1896], Wisselingh [1898] and Wester [1909], the cell-walls of the hyphal tissues of lichens contain no cellulose but are frequently composed of chitin; no chitin has, however, been found in *Cetraria islandica*, although it is met with in other members of the genus. The hyphal membranes of this lichen are, in fact, somewhat unusual, in being composed mainly, as Escombe showed, of lichenin and *iso*lichenin. The algal cell-walls were, on the other hand, considered by Escombe to be composed of a type of cellulose. Although it is as yet impossible to demonstrate that the hemicelluloses also occur in these gonidial walls, analogy with the higher plants suggests that such is the case. A small amount of the glucose synthesised by the algal cells becomes converted, by means at present unknown but presumably the same as those effective in the higher green plants, into galactose and mannose, thus giving the nucleus of the hemicelluloses.

In common with all northern lichens, *Cetraria islandica* has a very slow growth. The metabolic demands of the hyphae on the carbohydrate synthesised by the algal cells are thus small, with the result that the major part of the glucose undergoes condensation to lichenin and *iso*lichenin, which are deposited on the much-thickened hyphal walls. In view also of this general low rate of metabolism, oxidation of the carbohydrates deposited in the algal cell-walls only proceeds to a very slight extent, so that the uronic anhydride content of the hemicelluloses is low.

The explanation of the absence from the plant of pentosans and pectic substances probably lies in this low oxidation. In the pectins, oxidation of the sugar units has proceeded to a very considerable extent, some 80 % of the galactose having been oxidised to uronic anhydride; such compounds could only be formed in tissues where the oxidation processes are extensive. Norman [1929] remarks that "the formation of pectin in the plant appears to take place when metabolism is at its highest peak, that is, when growth is most rapid." Further it seems that reactions leading to pectin formation take place only in tissues where there is an abundant water supply. The principal habitat of *Cetraria* is in exposed places in sub-arctic regions; having no root system, it is dependent for its water supply on absorption over its whole surface during rain and storage within its tissues of the water so absorbed. Thus it is pre-

dominantly of xerophytic habit, and another of the conditions for pectin formation is lacking.

The pentosans are generally considered to arise from condensed uronic acids by some process of decarboxylation, either by mild oxidation [Norman and Norris, 1930] or other means. Whatever the means, the process does not take place in this lichen, since pentosans are virtually absent.

3. *The relation between pectins and hemicelluloses.*

The view has frequently been expressed that some at least of the hemicelluloses may arise in the higher plants by partial decarboxylation of the pectins. This suggestion has been based on the observation that in non-lignified tissues pectins predominate over hemicelluloses, while in older, lignified tissues, the reverse is the case, rather than on any direct observations of a change in the amounts of the two classes in the same tissue at different stages of growth. Other support for the view has been found in the production from pectin, *in vitro*, of substances resembling hemicelluloses in having a low uronic anhydride content [Candlin and Schryver, 1928; Norman and Norris, 1930]. So far as *Cetraria islandica* is concerned, however, the facts given in this communication do not support this view. Hemicelluloses containing galacturonic acid occur in fair quantity in the lichen, and if there had been any direct connection between these substances and pectic bodies, as regards their origin, then the large quantities of lichen employed, containing plant specimens of all ages, would have been expected to yield a small quantity of pectin at least; such was not the case. In addition, as no lignin was detected, any pectin present in the young tissues could not have been converted into this substance as has sometimes been suggested.

It might be argued that the absence both of pectins on the one hand, and pentosans and lignin on the other, could be regarded as evidence of a connection between these classes. This may indeed be the case. But the facts presented above make it necessary to emphasise the fact that not all polyuronides, of low uronic anhydride content, arise from pectins by decarboxylation. The only conclusion, then, is that the hemicelluloses of this lichen do not arise from pectic substances, but by feeble oxidation of condensed hexoses—a process distinct from, though largely parallel with, that which leads to the formation of pectins in plants whose general level of metabolism is higher.

Summary.

1. The cell-wall constituents of *Cetraria islandica*, other than lichenin and *iso*lichenin, have been shown to consist mainly of hemicelluloses and cellulose; pectins, pentosans and lignin are absent.

2. Three distinct hemicelluloses have been isolated, one of which is more closely associated with the cellulose and resists extraction with 4 % alkali.

3. All three hemicelluloses give, on hydrolysis, mannose, galactose and galacturonic acid; the amount of the last is in each case low (10 %).

4. Evidence is given to show that in two of the hemicelluloses, the molecule consists of a resistant acid nucleus, consisting of mannose, galactose and galacturonic acid units, and a more loosely combined portion, containing no mannose.

5. The relation between the chemical nature of the cell-wall constituents and the habit of growth of the lichen is discussed.

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CCXXXI. THE INFLUENCE OF VITAMIN C ON INTRACELLULAR ENZYME ACTION.

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THE discovery of glutathione by Hopkins [1921], and the recognition that it is the natural activator of certain intracellular proteolytic enzymes in animals as well as in plants [Waldschmidt-Leitz *et al.*, 1930; Grassmann *et al.* 1930; 1931], has established a relationship between these enzymes and the oxidation-reduction processes in the cell. In the ascorbic acid (vitamin C), isolated and crystallised from suprarenal gland, oranges and cabbage, Szent-Györgyi [1928; 1932; 1933] has recently found another constituent of the cell which assists in determining the oxidation-reduction potential and which may be reversibly oxidised or reduced.

Ascorbic acid in the organism is not limited solely to antiscorbutic action but has a still more comprehensive rôle. By its reversible oxidation and reduction, it is indirectly related to cell respiration and is also a determining factor in establishing the equilibrium between SH and SS compounds. The vitamin assumes a protective rôle against the oxidation of SH compounds in the organism[1]. The physiological significance of these two substances (glutathione and ascorbic acid) as catalysts of oxidation-reduction processes has been considerably increased through the discovery that the activity of certain intracellular enzymes is dependent on the presence of definite oxidation-reduction potentials.

Arginase is one of the most important enzymes of intermediary protein metabolism, whose activity, according to Waldschmidt-Leitz *et al.* [1933] and Edlbacher *et al.* [1925; 1927; 1932; 1933] is dependent upon oxidation-reduction potentials. This enzyme is also activated by the system, ascorbic acid *plus* iron as is shown by the following experiment.

Example. 0·25 cc. glycerol-liver-suspension (1:10), 4·5 cc. water, 10 cc. 1 % arginine carbonate, 5 cc. 0·1 *N* glycine buffer (p_H 9·5), incubated 60 minutes at 30°; addition, 20 mg. cysteine-HCl, previously neutralised, or 20 mg. crystalline ascorbic acid (prepared from oranges), or 0·5 cc. 0·1 *N* $FeSO_4$; incubated 60 minutes, p_H 7.

Addition	Arginase activity (cc. 0·02 *N* NH_3)
None	7·0
Iron	8·0
Cysteine-iron	15·0
Ascorbic acid (crystal pulp)	12·0
Ascorbic acid-iron	16·0

[1] This may explain the high concentration of sulphydryl compounds in the suprarenal gland.

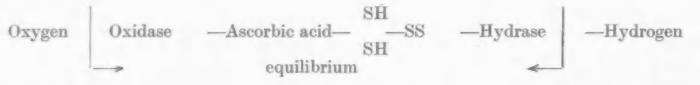
It is known that intracellular proteolytic enzymes of the cathepsin type require the presence of sulphydryl compounds for their activation. Ascorbic acid, by virtue of its oxidation-reduction potential, also apparently regulates the relation of SH to SS as is shown by these experiments. The decomposition of proteins is thus dependent on the presence of vitamin C. The conception, therefore, of a regulation of the intracellular protein metabolism by the interaction of these two substances, glutathione and ascorbic acid, in the sense of hydrolysis on the one hand and of synthesis on the other, possesses a physiological significance. It remains to be determined [*cf.* Grassmann *et al.* 1931] whether sulphydryl compounds alone are responsible for the activation of the catheptic enzyme systems.

Example. 5 cc. carcinoma-glycerol suspension (1:10), 0·40 g. gelatin, incubated 24 hours at 30°, p_H 4·0, total volume 25 cc. Results given are for 10 cc. of the mixture.

Additions	NH_2 increase (cc. 0·05 *N* KOH)
None	0·75
Cysteine (20 mg.)	1·70
Ascorbic acid (20 mg. crystal pulp)	1·60

There is scarcely a doubt that, in addition to the catheptic enzymes and arginase, other intracellular enzymes require the participation of these two substances for their activation. Among the enzyme groups which we are investigating from this point of view in our laboratories, methylglyoxalase is of particular interest. If it should turn out that the action of this enzyme is related to the oxidation-reduction potential in the cell, a new explanation would be found for the excess glycolysis in anaerobically developing cells, *e.g.* in malignant tumours.

These findings, by which for the first time a relationship is established between a vitamin and the intracellular enzymes of metabolism, are of particular significance for the question of the cause of autolysis in dying cells, which plays such an important part in malignant tumours. The much disputed question of the formation and occurrence of sulphydryl compounds in living and dying cells is perhaps plausibly explained by the following working hypothesis for respiration.



The solution of this question in connection with the activation relationships of intracellular enzyme systems, particularly those of the carbohydrate-degrading enzymes in healthy muscle and malignant tumours, will be the object of further experiments.

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CCXXXII. PHENYL *ISO*CYANATE PROTEIN COMPOUNDS AND THEIR IMMUNOLOGICAL PROPERTIES.

II. THE GELATIN COMPOUNDS.

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Native proteins, with a few exceptions, when injected into the animal body readily produce antibodies. By a comparison of the chemical constitution and properties of various proteins it is possible to say that the presence of a certain amino-acid or grouping is not essential for antigenic power, and, in some instances, to offer a feasible explanation for the non-antigenicity of a certain protein. Thus, although zein is deficient in tryptophan and lysine, and caseinogen in cystine, these two proteins are markedly antigenic, and the conclusion can be reached, therefore, that the three amino-acids mentioned are not essential for antigenicity. The protamines [Wells, 1913; *cf.* review by Wells, 1929], gelatin [Wells, 1908; 1916; Landsteiner, 1917; Starin, 1918; Kahn and McNeil, 1918] and the closely related preparation, glue [Ramsdell and Walzer, 1927], on the other hand, are non-antigenic. Gelatin appears to be of special interest in this respect, and the non-antigenicity of this protein has attracted much attention. Starin [1918], in particular, carried out an extensive investigation, using the precipitin, anaphylactic, complement fixation and meiostagmin reactions, and decided that the injection of gelatin into rabbits, guinea-pigs and dogs failed to produce antibodies to gelatin. This failure of gelatin to incite antibody production has been interpreted in several ways, but the view most commonly held suggests that the non-antigenicity in this instance is due to the absence of aromatic groupings, for gelatin is deficient in tyrosine and tryptophan, and it contains only a very small amount of phenylalanine. Gelatin, however, differs from other proteins in many respects, and several additional suggestions might be advanced to account for its peculiar immunological properties. Thus, it is important to know whether the failure to produce antibodies is entirely due to the absence of aromatic groupings, and for this purpose it was decided to introduce into the gelatin molecule groupings of this nature.

Landsteiner [1919] found that gelatin coupled with diazotised metanilic acid gave precipitin reactions with antisera produced by the injection of horse-serum-proteins coupled with *m*-diazobenzenesulphonic acid, and that in higher concentrations the gelatin compound specifically inhibited the precipitin reaction between these azoproteins and their antisera. Adant [1930] and Bruynoghe and Vassiliadis [1930] studied the serological properties of gelatin coupled with diazotised aniline and obtained some antibody formation when this preparation was injected. The results of these authors will be discussed more fully later on in this paper. Medveczky and Uhrovits [1931], working with benzoylated proteins, have shown that immune sera produced by the injection of the benzoylated

proteins of horse serum or of benzoylated typhoid bacilli will give good precipitin reactions with benzoylated gelatin, and that the injection of benzoylated gelatin produces anaphylaxis in animals sensitised with benzoylated typhoid bacilli. Hooker and Boyd [1933] have very recently studied the immunological properties of gelatin coupled with diazotised arsanilic acid, and have found that the injection of this preparation produces sera which give precipitin and complement fixation reactions with other proteins coupled with diazotised arsanilic acid but not with the homologous antigen (gelatin-diazo-arsanilic acid). The evidence so far available appears to show, therefore, that gelatin coupled with these new groupings can take part in precipitin and similar reactions, but evidence of the acquirement of true antigenic function does not appear to be very satisfactory.

In a previous paper [Hopkins and Wormall, 1933] the immunological and chemical properties of phenylureidoproteins have been studied. These compounds are prepared by the action of phenyl *iso*cyanates on various proteins, a reaction used by Raper [1907] for the preparation of phenylureidopeptone compounds. By this reaction, a new grouping, $C_6H_5NH.CO—$ or a related group, is introduced into the protein molecule, and in the earlier paper evidence has been presented which strongly suggests that the introduction occurs mainly, if not entirely, at the free amino-groups of the lysine molecules. The reaction can be represented in the following manner:

$$\begin{matrix} R_1.CH.NH_2 \\ | \\ R_2 \end{matrix} + OC{:}N.C_6H_5 \rightarrow \begin{matrix} R_1.CH.NH.CO.NH.C_6H_5 \\ | \\ R_2 \end{matrix}$$

In this way, aromatic groupings are introduced into the protein, and the mode of linkage is not very different from the ordinary peptide linkage which appears to link up most of the amino-acids in the protein molecule. This reaction thus offers an alternative method for studying the effect of the introduction of new groups into the protein molecule, and for several reasons it is most suitable for this purpose. The mode of linkage is not unlike that already present and the protein is not subjected to very drastic treatment. Furthermore, it appears very desirable that different methods of introducing the same new group into different parts of the same protein molecule might be studied. Experiments along these lines are being carried out and will be reported later. In the investigation described here, a study has been made of the chemical and immunological properties of phenylureido- and *p*-bromophenylureido-gelatin, the primary object being to determine whether the introduction of aromatic groupings into the gelatin molecule would render it fully antigenic. For this purpose these gelatin compounds have been tested, in precipitin and complement fixation tests, with several antisera against phenylureido-horse-serum-globulin, and immunisation experiments have been carried out to determine whether antibodies are produced when these phenylureidogelatin compounds are injected into rabbits.

Experimental.

Preparation of phenylureidogelatin.

4 g. of gelatin (Coignet's "Gold Label") were dissolved in 400 cc. of warm water, 200 cc. of a phosphate buffer of p_H 8·0 added, the mixture cooled and stirred and 3·2 cc. of phenyl *iso*cyanate added. The mixture was kept at p_H 8 and stirred for about 1 hour. The phenylureidogelatin was then precipitated by acetic acid and purified by two further precipitations as described in a previous

paper [Hopkins and Wormall, 1933]. This amount of gelatin usually furnished under these conditions about 60–80 cc. of a solution containing about 2 % of protein. NaCl was added to give 0·9 %, and the solution was filtered through a Berkefeld filter-candle. The filtered solution was kept in sterile tubes in the ice-chest, and the solidified mass was melted by gentle heat when required.

The solutions thus obtained were perfectly clear and closely resembled solutions of gelatin. The phenylureidogelatin compounds, like the similar compounds of serum-globulin and caseinogen, can be precipitated by the addition of dilute acetic acid to p_H 4–4·5, and the precipitates are readily soluble again at p_H 7·5, although with the gelatin preparations dissolution is best effected by warm water or NaCl solution (25–30°). The solutions used in this work (containing 1–2·5 % of phenylureidogelatin) solidified on cooling, and thus substitution in the free amino-groups of the gelatin molecule does not destroy the capacity to form gels. In this connection, it is interesting to note that gelatin treated with nitrous acid or with formaldehyde still exhibits gel-formation.

Preparation of p-*bromophenylureidogelatin.*

This compound was prepared by the addition of a filtered ethereal solution of *p*-bromophenyl *iso*cyanate (4 g. in about 30 cc. of ether) to a cooled and stirred mixture of 400 cc. of 1 % gelatin (Coignet's "Gold Label") and 200 cc. of phosphate buffer of p_H 8·0. The mixture was cooled, kept at p_H 8 and stirred for about 1½ hours, after which precipitation and purification were effected as described previously [Hopkins and Wormall, 1933]. From this amount of gelatin, 50–60 cc. of a solution containing about 2·5 % of *p*-bromophenylureidogelatin were usually obtained. NaCl was added to the extent of 0·9 % and the solution filtered through a Berkefeld filter-candle and kept in the ice-chest.

Nitrogen determinations.

The ratios of free amino-N to total N in the above preparations and in gelatin were determined, and in this way a measure of the amount of substitution was obtained, as described in the previous paper. The main bulk of the antigen solutions used for the immunisation experiments gave the following figures for the free amino-N (Sørensen's formaldehyde method) calculated as a percentage of the total N: gelatin, 3·0 %; phenylureidogelatin, 0·8 % and *p*-bromophenylureidogelatin 1·2 %. These figures indicate substitution to the extent of 70–75 % in the phenylureido-compound and about 60 % in the *p*-bromophenylureidogelatin. The last-named compound was found to contain 2·2 % of bromine, whilst another preparation of the same substance had a bromine content of 2·6 %.

Immunisation.

Three groups, each of 4 rabbits, were used for the injection of the following substances:

Group (*a*) Gelatin (Coignet's "Gold Label").
Group (*b*) Phenylureidogelatin.
Group (*c*) *p*-Bromophenylureidogelatin.

The control injections with untreated gelatin were made in order to establish the non-antigenicity of the sample of gelatin used in this investigation. The two phenylureidogelatin compounds were prepared as described above. It was thought desirable in these experiments, where the introduction of aromatic

groupings was being studied, to avoid the use of phenol as antiseptic, and therefore all the solutions used for injection were sterilised by filtration through Berkefeld filter-candles.

At each injection the rabbits received a volume of the antigen solution containing 0·25 g. of the protein, and the injections were made at intervals of 7 or 8 days. These injections were usually made intraperitoneally, but with 50 % of the animals the last two injections were intravenous. Samples of serum from each rabbit were tested 7 or 8 days after the third, fourth, fifth, sixth and seventh injections and also about 3 weeks after the seventh injection. Precipitin tests were carried out on each occasion, and usually complement fixation tests were also made. Each serum was tested against all three antigens (gelatin, phenylureidogelatin and *p*-bromophenylureidogelatin) and against phenylureido-chicken-serum-proteins and phenylureido-rabbit-serum-proteins.

Precipitin tests. The technique of these tests was as described previously [Johnson and Wormall, 1930; Hopkins and Wormall, 1933]. Some of the tests were made at 37°, but the majority were done at room temperature (15–20°), since it was found early in this work that the gelatin antigens gave much better precipitin reactions at the lower temperatures. In many instances tests were made at 37° as well as at room temperature. The results were recorded as follows: (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++ (increasing degrees of precipitation).

Complement fixation tests. These were carried out as described previously [Johnson and Wormall, 1930], the results being recorded as follows: 4 (complete haemolysis), − (no haemolysis), 3, 2 and 1 (intermediate degrees of haemolysis).

(1) *The phenylureidogelatin compounds and their reactions with antisera to phenylureido-horse-serum-globulin.*

The preliminary observation that phenylureidogelatin preparations give precipitates with antisera to phenylureido-horse-serum-globulin, noted in a previous paper [Hopkins and Wormall, 1933], was amplified by making tests with various antisera and using much wider ranges of antigen dilution. These precipitin reactions were also compared with those obtained in similar series of phenylureido-horse-serum-proteins and phenylureido-chicken-serum-proteins tested against the same antisera. Precipitin tests were made both at 37° and at room temperature, and from the results obtained (*cf.* Table I) the conclusion was reached that room temperature is preferable to 37° for precipitin tests with the gelatin antigens. Indeed it has frequently been observed that well-marked precipitates of phenylureidogelatin *plus* antisera to phenylureido-horse-serum-globulin have become much less bulky after incubation at 37°. This was well illustrated in the experiment quoted in Table I, for on the addition of the antiserum to phenylureidogelatin good precipitates were obtained, and these became much less pronounced after 1 hour at 37° and still less marked after 3 hours at this temperature. When these antigen-antibody mixtures at 37° were cooled, the gelatin antigen precipitates became stronger again and were comparable with those at room temperature. Thus the precipitates of "phenylureido"-antiserum with phenylureidogelatin appear to be more soluble at 37°, possibly owing to the retention by the gelatin of some of its solubility properties. The other antigens used in these tests, phenylureido-chicken-serum-proteins and phenylureido-horse-serum-proteins behaved in the normal manner and with antisera to phenylureido-proteins gave precipitates at 37° which were equal to, or greater than, those obtained at room temperatures.

Table I. *Comparison of the precipitin reactions with various phenylureido-protein compounds and the influence of temperature on these reactions.*

		Immune serum (anti-phenylureido-horse-serum-globulin)					
		No. 50		No. 51		No. 53	
Antigen	Antigen dilution	16°	37°	16°	37°	16°	37°
Phenylureidogelatin	1 : 20	–	–	–	–	–	–
	1 : 100	f.tr.	–	tr.	f.tr.	tr.	–
	1 : 500	±	tr.	+	±	+	±
	1 : 2500	+	±	+	±	+	±
	1 : 12,500	tr.	tr.	tr.	tr.	tr.	tr.
Phenylureido-chicken-serum-proteins	1 : 20	±	±	+	+	±	+
	1 : 100	+	+	+	+	+	+
	1 : 500	+	+	+	+ ±	±	±
	1 : 2500	tr.	tr.	±	±	tr.	tr.
	1 : 12,500	–	–	–	–	–	–
Phenylureido-horse-serum-proteins	1 : 20	+ +	+ +	+ + ±	+ + ±	+ ±	+ ±
	1 : 100	+ + ±	+ + ±	+ + ±	+ + ±	+ +	+ +
	1 : 500	+ ±	+ ±	+ ±	+ ±	+	+ ±
	1 : 2500	±	±	±	±	tr.	±
	1 : 12,500	–	–	–	f.tr.	–	–

The results given in Table I indicate a very marked capacity on the part of phenylureidogelatin to give precipitates with antisera to phenylureido-horse-serum-globulin, but maximum precipitation is observed with much greater dilutions of the gelatin preparations compared with the similar preparations of horse-serum-proteins or chicken-serum-proteins. These differences in the zone of maximum precipitation may be related to the number of reactive groupings in the antigen, but this does not appear probable since the preparations used for these tests (Table I) did not differ to any great extent in the number of phenylureido groupings introduced. Complement fixation tests have also demonstrated the same difference in capacity to react with the antiserum, the phenylureidogelatin preparations being active in much higher dilutions than are the corresponding serum-protein compounds (*cf.* Table II).

Table II. *Complement fixation tests with phenylureidogelatin compounds.*

(Tests with immune serum, No. 51—anti-phenylureido-horse-serum-globulin.)

	Antigen dilution									Reading after (hr.)
Antigen	1:20	1:60	1:180	1:540	1:1620	1:4860	1:14,580	1:43,740	Control (NaCl)	
Phenylureido-horse-serum-proteins	4	3	—	—	—	—	1	4	4	½
	4	3	1	—	—	—	2	4	4	1
Phenylureido-gelatin	4	2	—	—	—	—	—	—	4	½
	4	4	1	—	—	—	—	—	4	1

The other gelatin preparation used in this investigation, *p*-bromophenylureidogelatin, gave very similar results with all the antisera, but the precipitin and complement-fixation reactions with this preparation were not quite so pronounced as those with phenylureidogelatin.

Inhibition tests, involving the addition of simple substances to mixtures of the antigen and antibody in order to determine which groupings could specifically inhibit the formation of the precipitate [Landsteiner, 1920; Landsteiner and van der Scheer, 1931; 1932] were made with certain amino-acids and their

phenylureido-compounds in a previous paper [Hopkins and Wormall, 1933]. These experiments, made with phenylureido-chicken-serum-proteins and antisera to phenylureido-horse-serum-globulin, furnished very strong evidence for the view that the immunologically active grouping in the phenylureidoprotein is the phenylureidolysine group. Similar results have been obtained from tests carried out with phenylureido- and *p*-bromophenylureido-gelatin as antigens in the precipitin reaction. Experiments have also been made to determine whether similar inhibition might be obtained in complement fixation tests with the phenylureidogelatin compounds, and the results of an experiment of this nature are given in Table III. From these results it will be seen that phenylureidolysine, and to a lesser extent phenylureidoalanine, inhibit these complement fixation reactions.

Table III. *Inhibition of complement fixation tests.*

Antiserum. No. 51 (anti-phenylureido-horse-serum-globulin).
Antigen. Phenylureidogelatin.

Antigen dilution	Inhibiting substance: NaCl		Lysine		Alanine		Phenylureido-lysine		Phenylureido-alanine	
1 : 20	4	4	4	4	4	4	4	4	4	4
1 : 60	4	4	4	4	4	4	4	4	4	4
1 : 180	2	3	3	4	2	3	4	4	4	4
1 : 540	—	—	—	2	—	—	4	4	3	4
1 : 1620	—	—	—	—	—	—	4	4	1	2
1 : 4860	—	—	—	—	—	—	4	4	—	1
1 : 14,580	—	—	—	—	—	—	4	4	—	2
1 : 43,740	—	—	—	—	—	—	4	4	2	3
1 : 131,220	1	3	1	2	1	3	4	4	4	4
1 : 393,660	3	4	2	3	3	4	4	4	4	4
Control (NaCl)	4	4	4	4	4	4	4	4	4	4
Reading (hr.) after	½	1	½	1	½	1	½	1	½	1

Details of tests. A mixture of 0·5 cc. of the antigen, 0·10 cc. of the inactivated antiserum, 0·10 cc. of 1 : 5 guinea-pig serum and 0·10 cc. of a neutral *M*/100 solution of the "inhibiting" substance (in 0·9 % NaCl solution) was kept at room temperature for 1 hour. 0·5 cc. of a 4 % suspension of sensitised ox red cells was then added to each tube and the tubes placed in a water-bath at 37°.

(2) *Immunisation experiments with phenylureido- and p-bromophenylureido-gelatin.*

In order to determine whether the introduction of aromatic groupings into gelatin really endows this protein with antigenic power, immunisation experiments have been carried out with phenylureidogelatin and *p*-bromophenylureidogelatin. In these experiments the modified gelatins were injected into rabbits as described earlier in this paper, and the sera of these rabbits were tested frequently by precipitin and complement fixation tests, for the presence of antibodies to gelatin itself, to phenylureidogelatin or to any other phenylureidoprotein (phenylureido-chicken-serum-proteins, *etc.*). Each rabbit received seven injections of the phenylureido- or *p*-bromophenylureido-gelatin, at intervals of 7 or 8 days, and the sera were tested 7 or 8 days after the third and subsequent injections. Throughout the whole of each series of injections the serological tests gave negative results, although there were one or two occasions when the precipitin tests made with these sera and phenylureido-chicken-serum-proteins (or phenylureido-rabbit-serum-proteins) showed very faint traces of a precipitate. These turbidities were very slight, however, certainly not more

significant than a faint trace, and they were only observed in very strong antigen solutions (a 1 : 20 solution of the 5 % protein solution). Since even these slight reactions were never obtained with the concentrations of antigen normally used for the testing of antisera, and since they were obtained on a few occasions only, the conclusion was reached that they are not significant. The complement fixation tests made simultaneously with the same sera and the same antigen solutions were completely negative on every occasion.

Discussion.

The action of phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate on gelatin results in the production of phenylureidogelatin compounds which readily give precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin. In the complement fixation tests these gelatin compounds appear to behave very much like the corresponding serum-protein compounds, but in the precipitin reactions they show certain significant differences. The chief difference is related to the influence of temperature on the extent of precipitation, the amount of precipitate formed with the gelatin compound being much less at 37° than at 16–20°. This difference is possibly related to the greater solubility of gelatin at the higher temperature. A comparison of the precipitin and complement fixation tests with phenylureidogelatin and the corresponding serum-protein compounds has also shown that the zone of maximum precipitation, or the zone of complete complement fixation, is found in much more dilute solution with the gelatin compounds. This difference in activity does not appear to be related to the number of phenylureidolysine groupings in the phenylureido-protein, and at the present time no explanation can be advanced to account for the greater power of the gelatin compounds to give precipitates in very dilute solution.

This ability to give precipitates or complement fixation when mixed with various antisera does not in any way prove that the phenylureidogelatin preparations are truly antigenic. A similar power to give precipitation with suitable antisera is exhibited by the soluble specific substances, which do not normally incite antibody response when injected into an animal [Heidelberger and Avery, 1923, *cf.* Review by Heidelberger, 1927]. More recent work suggests that some modification of the view that these immunologically active carbohydrates and similar haptens are non-antigenic, will perhaps be necessary, since it has been shown that certain of these haptens can produce antibodies when injected after adsorption on kaolin, collodion particles, charcoal, *etc.* [Gonzalez and Armangue, 1931; Zozaya, 1931; 1932; Landsteiner and Jacobs, 1932; 1933]. Any modification of this view does not affect, however, the argument advanced here that the capacity to give a precipitate or to give complement fixation when certain antisera are added does not necessarily imply that the substance is antigenic, *i.e.* that it will produce antibodies when injected into an animal. There are several other instances of precipitin reactions with substances which are not truly antigenic. Thus, antibodies to azoproteins will give precipitates with azo-dyes [Landsteiner and van der Scheer, 1932], indicating the specific combination of antibodies with substances of relatively small molecular weight to give a precipitate. The demonstration of true antigenic power involves the formation of antibodies when the substance is injected into an animal, these antibodies being detected by the precipitin, complement fixation or some similar test.

The immunisation experiments with phenylureido- and *p*-bromophenyl-ureido-gelatin compounds described in this paper have shown that these com-

pounds do not produce any significant antibody response detectable by precipitin or complement fixation reactions. From these results the conclusion can be reached, therefore, that to render gelatin antigenic in the full sense it is not sufficient to introduce aromatic groupings. The non-antigenicity of gelatin, although it might conceivably be due in part to the absence of such groupings, is not due solely to this deficiency. For several reasons it would be desirable to introduce into the gelatin molecule an aromatic grouping which is completely or almost completely identical with one of those present in other proteins, *e.g.* the tyrosine or tryptophan groupings linked to the rest of the molecule by means of a peptide linkage. It is hoped that experiments along these lines will be made in the near future, but it is realised that it may be extremely difficult to effect the introduction of a grouping of this kind without recourse to a fairly drastic method which will involve some other change in the protein molecule.

The results obtained by Adant [1930] and Bruynoghe and Vassiliadis [1930] are difficult to correlate with those recorded in this paper. Adant found that gelatin coupled with diazotised aniline produced, when injected into the rabbit, antibodies which would give precipitates with gelatin-diazotised aniline and also with gelatin itself. The reaction between these antisera and gelatin is most difficult to explain, and it appears necessary to postulate the acquirement of antigenic power when diazotised aniline is coupled with gelatin and the formation of at least two types of antibody or antibody groupings when this complex is injected; one antibody which can specifically react with any diazotised aniline-protein compound and another which can specifically react with gelatin. Bruynoghe and Vassiliadis [1930] carried out complement fixation tests with these antisera to gelatin-diazotised aniline and obtained fixation with gelatin and not with gelatin-diazotised aniline. These findings also are difficult to interpret and without further experimental details it would be impossible to assess the significance of these complement-fixation results. Hooker and Boyd [1933] have recently carried out similar investigations using gelatin-diazotised arsanilic acid, and they find that this gelatin compound produces in the rabbit antibodies which will give precipitin reactions with other proteins coupled with diazotised arsanilic acid but not with gelatin-diazotised arsanilic acid or with gelatin. These results of Hooker and Boyd indicate that antigenicity is to some extent conferred on gelatin by coupling it with diazotised arsanilic acid, but, as these authors point out, the results "do not show that the non-antigenicity of gelatin is due solely to its deficiency in aromatic amino-acids." Hooker and Boyd record the significant finding, but one which is difficult to interpret, that gelatin-diazotised arsanilic acid reacts with antisera to egg-white-diazotised arsanilic acid but not with antisera to gelatin-diazotised arsanilic acid, although the last-named antisera give precipitates with egg-white proteins (or caseinogen) coupled with diazotised arsanilic acid. From many points of view, it might be claimed that the method used in the work described here has several advantages over other methods which have been used for the introduction of aromatic groupings into the gelatin molecule. With the phenyl *iso*cyanate method, the linkage is one which does not differ very considerably from the ordinary peptide linkage, and a second point in its favour is that any excess of phenyl *iso*cyanate is rapidly destroyed and the products formed from it are easily removed from the phenylureidogelatin solution.

From the immunisation experiments described here, it appears probable that some explanation other than that relating to aromatic groupings will have to be advanced to account for the non-antigenicity of gelatin, but any suggestion offered at the present time will be mainly speculative. The chemical constitutions

of gelatin and other proteins can be compared and the non-antigenicity of the former attributed to the absence of some substance or grouping which is present in all antigenic proteins. Gelatin is deficient in, amongst other things, aromatic amino-acids and carbohydrate groupings. The mere introduction of benzene rings does not appear to convert gelatin into an antigen, and with reference to the second possibility there appears to be little or no evidence that proteins must contain a carbohydrate grouping before they are antigenic. The carbohydrates associated with certain bacterial proteins undoubtedly play the predominant rôle in the determination of certain types of specificity, and there are probably very many instances of specificity determined by the carbohydrates present. It does not necessarily follow, however, that protein specificity is always determined by the carbohydrate groupings which are usually associated with native proteins, nor does it mean that antigenicity is exhibited only by those proteins which contain carbohydrate groupings. In this case, however, as with the other suggestions which might be offered to account for the non-antigenicity of gelatin, it does not appear profitable at the present time to discuss the matter at any length, and much additional evidence will be needed before it is possible to reach any final decision.

Summary.

1. Phenylureido- and *p*-bromophenylureido-gelatin have been prepared by the method previously described. Some properties of these compounds are described.

2. These gelatin preparations give marked precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin.

3. With the gelatin preparations the zone of maximum precipitation is found in higher dilutions than are the corresponding zones for phenylureido-compounds of horse-serum-globulin or of caseinogen. This difference does not appear to be related to the number of new groupings introduced.

4. The precipitin reactions with these gelatin compounds are most pronounced when carried out at room temperature (15–20°) or at lower temperatures, since there is a strong tendency for the precipitates to go into solution at 37°.

5. Phenylureido- and *p*-bromophenylureido-gelatin when injected into rabbits over a long period fail to produce antibodies which are detectable by precipitin and complement fixation tests. The conclusion is reached, therefore, that the non-antigenicity of gelatin is not due solely to a deficiency in aromatic groupings.

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CCXXXIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

XII. THE PREPARATION AND METABOLISM OF *d*-ACETYLCYSTEINE.

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The racemising action of acetic anhydride on amino-acids has attracted a considerable amount of attention recently. For this reason and also because du Vigneaud and Sealock [1932] have suggested that the acetylcysteine made by one of us [Pirie, 1931, 1] was partly racemic it seemed advisable to study this substance further. The metabolic work which we carried out with this doubtful material [Hele and Pirie, 1931] had also to be repeated.

Using the method of preparation already described, very little racemisation does in fact occur; the preparation has, however, been simplified by the substitution of lead acetate for cuprous oxide as a precipitant for the acetylcysteine. The use of ketene as an acetylating agent was suggested to us by the work of Bergmann and Stern [1930]. The reaction proceeds very smoothly in alkaline solution and gives, as is shown later, an unracemised product in nearly quantitative yield.

The fact that the cysteine in mercapturic acid is acetylated gives a special interest to metabolic studies on acetylcysteine. The results of previous papers on the metabolism of related compounds [Hele and Pirie, 1931; Pirie, 1932] may be summarised as follows:

Substance	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Cysteine or cystine	70	4
Glutathione	72	10
Glycylcysteine	56·5	19·5
Methionine	66	16

The present work on acetylcysteine gives the following mean figures. After oral administration 48 % of the dose is excreted as sulphate and 32 % as neutral sulphur; after subcutaneous administration the figures are 28 and 42 %. Our previous results with orally administered acetylcysteine were similar but on the single occasion when it was given subcutaneously a larger percentage was oxidised. It is clear that acetylation has reduced considerably the oxidisability of the molecule.

Preparation of acetylcysteine. A brisk current of ketene, prepared by the method of Ott *et al.* [1931], is passed into a cold suspension of 20 g. of cystine in 130 cc. of 0·6 *N* NaOH. The mixture is kept cool with tap water and after

half an hour 30 cc. of 2·5 *N* NaOH are added. All the cystine soon goes into solution and after about an hour the action is complete, *i.e.* a sample has no formaldehyde titration. The solution is now slightly acid, 20 cc. of glacial acetic acid and some zinc are added and the mixture warmed to 50–60° and allowed to cool. It is stirred occasionally and filtered when cold.

The filtrate is made up to about 400 cc., and normal lead acetate solution is added till the supernatant fluid after centrifuging a sample gives no further precipitate with more lead acetate. The precipitate is centrifuged and washed two or three times on the centrifuge with 1 % lead acetate solution; it is then suspended in water and decomposed with hydrogen sulphide. There should be no avoidable delay during the operations described in this paragraph or a brown colour will develop, and the yield will be small.

The lead sulphide is removed by filtration and the filtrate distilled to small bulk *in vacuo*. Acetylcysteine crystallises readily in thick prisms and occasionally in well-formed octahedra; yield 21·5 g.

The product made in this way is purer than that obtained by the old method; its M.P. is 111° instead of 107–109°, and a 2·7 % solution in water has $[\alpha]_{546}^{21°} = +6{\cdot}3°$.

1·28 g. of this *d*-acetylcysteine were hydrolysed by boiling for 2 hours with 20 cc. of 6 *N* HCl and evaporated to dryness. The residue was taken up in water, neutralised and aerated after adding a trace of iron. 0·95 g. of cystine was obtained; this is 94 % of the theoretical amount. A 0·4 % solution in *N*/9 HCl showed $[\alpha]_{546}^{20°} = -274°$. This value is that to be expected for a dilute solution of cystine in weak acid [Pirie, 1931, 2]; it is clear therefore that, when prepared by the ketene method, acetylcysteine undergoes little or no racemisation.

If a ketene lamp is not available an almost equally unracemised product may be made, though rather less conveniently, with acetic anhydride as in Nicolet's [1930], Hollander and du Vigneaud's [1931] and Pirie's [1931, 1] methods if the reaction mixture is kept cold. The reduction and precipitation with lead are carried out exactly as in the method described in this paper. A sample of acetylcysteine prepared in this way was hydrolysed with 6 *N* HCl: the cystine obtained from it had $[\alpha]_{546}^{20°} = -265°$. Under these conditions therefore racemisation, as might be expected from the work of Bergmann and Zervas [1928], does not occur very readily.

Diacetylcystine is easily prepared from acetylcysteine by oxidation with hydrogen peroxide. Experiments carried out in the same manner as those already published by one of us [Pirie, 1931, 2; 1933] show that this oxidation is catalysed by iron and thiocarbamide in much the same way as the oxidation of glutathione, but that copper has only a slight catalytic power. The oxidation is therefore carried out in the same way as that of glutathione [Pirie, 1931, 1] but using a trace of iron as catalyst. Diacetylcystine prepared in this way, like that prepared by Hollander and du Vigneaud [1931], is a non-crystalline, slightly gummy solid, exceedingly soluble in water.

Metabolic experiments.

Two bitches, Patsy (8·5 kg.) and Christina (8·7 kg.), were used. The diets and analytical methods were the same as in our earlier work [Hele and Pirie, 1931]. When fed in the amounts used in this work *d*-acetylcysteine showed no signs of being poisonous and did not affect the dog's nitrogen excretion appreciably.

The results of nine experiments are as follow.

	Dog	Dose g.	Route	% of dose excreted as sulphate in 2 days	% excreted as neutral sulphur	% of dose accounted for
1.	Patsy	1·63	Orally	34	32	66
2.	,,	,,	,,	59	35	94
3.	,,	,,	,,	56	31	87
4.	Christina	,,	,,	46	39	85
5.	,,	,,	,,	40	31	71
6.	,,	,,	,,	47	30	77
7.	,,	,,	Subcutaneously	29	39	68
8.	,,	0·815	Orally	52	27	79
9.	,,	1·63	Subcutaneously	27	46	73

In Exps. 1, 3, 5 and 9 *d*-acetylcysteine was prepared by the old method and in the others by the ketene method.

The rise in neutral sulphur observed after feeding acetylcysteine is due, in part at least, to the excretion of that substance unaltered in the urine. From a mixed batch of urines passed on the day following the dose an amount of acetylcysteine corresponding to one-fifth of the rise in neutral sulphur was isolated. The method of isolation (precipitation with acid mercuric sulphate followed by the removal of sulphuric acid from the decomposed mercury precipitate and precipitation with lead acetate) could not, however, be expected to give even an approximately quantitative recovery.

A sample of each day's urine was reduced with zinc and sulphuric acid and titrated with *N*/100 iodine. The titration figure agreed closely with the rise in neutral sulphur on each dose day, but we feel that the actual value has little quantitative significance.

Summary.

d-Acetylcysteine can be conveniently prepared by the action of ketene on cystine in alkaline solution.

When *d*-acetylcysteine is fed to a dog 48 % is excreted as sulphate and 32 % as neutral sulphur. It is less readily oxidised when given subcutaneously.

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CCXXXIV. THE DETERMINATION OF THE ANTINEURITIC VITAMIN.

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PART I. PIGEONS.

The methods employed at the present time for the estimation of the antineuritic vitamin are varied. Pigeons have long been used in tests in which the vitamin is administered (*a*) to prevent head retraction, (*b*) to cure head retraction and (*c*) to maintain body weight. Recently Kline *et al.* [1932] have described the use of chicks. Rats have been used in growth tests and in tests in which paralytic symptoms are cured [Smith, 1930]. Mice have also been recommended by Freudenberg and Cerecedo [1931] for growth tests.

Since the number of different factors discovered to be present in the commoner sources of the antineuritic vitamin continues to increase, methods of estimation in which an antineuritic effect is produced have an advantage over tests depending on growth or weight maintenance. Further an element of doubt remains whether the principle which cures the symptoms of paralysis in rats is identical with the antineuritic principle of Eijkman. It is true that Smith [1930] found that concentrates prepared by Seidell which prevented the onset of symptoms in pigeons were able to cure the symptoms in rats, but the ratio between the pigeon maintenance dose and the curative rat dose was not the same in different preparations. We feel that the claim of a method, in which birds are not used, to measure the antineuritic vitamin must rest ultimately on evidence that results obtained by that method are parallel with results obtained by a method in which birds are used.

Tests on pigeons must in our opinion be curative tests; as Smith has pointed out, a preventive test such as that of Williams [1916; 1917] takes too long, and a test like that of Seidell [1922], in which maintenance of body weight is the criterion, cannot be assumed to be a test for the antineuritic vitamin. It has, however, been difficult to find a curative test which gives trustworthy results. Kinnersley *et al.* [1928] have discussed the curative test in detail and base their procedure on the view "that there is fundamentally a relation between the amount of the factor supplied and the time of protection after cure."

When a pigeon develops head retraction after being fed on polished rice, Kinnersley *et al.* administer a dose of the preparation to be tested; if no relief of symptoms is seen, they give a further dose; they observe the number of days for which the pigeon remains cured. If the total dose of the preparation administered is x g., and if the duration of the cure is y days, they express the

potency of the preparation as y/x "day doses per g." The average result from several birds is taken as the true result.

As the work in this laboratory involves many routine examinations, we have been attracted to the curative test for pigeons because of the simplicity of the test; it involves less work and skilled attention than the rat test described by Smith, or indeed than any other vitamin test. We have accordingly investigated the response of pigeons suffering from head retraction to different doses of several preparations containing vitamin B_1, testing the effect of each dose of a preparation upon several pigeons. The investigation has been continued during 31 months in which 688 birds have been given a diet of polished rice and water; as a result we are proposing a different method of using pigeons which we believe makes it possible for more accurate results to be obtained than are given by the day-dose method in routine tests.

Details of the treatment of the pigeons.

The pigeons were obtained from a dealer in groups of 30 at one time and were birds weighing 300–450 g. We did not give them a stock diet for a preliminary period but fed them on polished rice at once; nor did we wash the rice. Out of the total number of 688 birds used by us, head retraction occurred in 310 (45 %). The figures for different months are given in Table I and show

Table I. *Apparent seasonal variation in percentage of pigeons developing head retraction on a polished rice diet.*

	Total no. of birds given polished rice diet			Percentage of birds in which symptoms occurred in 30 days		
	1931	1932	1933	1931	1932	1933
January	30	—	32	23	—	19
February	30	32	32	40	42	69
March	23	32	30	40	53	60
April	32	30	—	50	47	—
May	32	24	30	41	37	57
June	32	—	—	56	—	—
July	20	—	24	45	—	37
August	—	—	—	—	—	—
September	31	—	—	55	—	—
October	32	32	—	53	47	—
November	32	32	—	59	34	—
December	32	32	—	28	42	—

that the proportion was lower in January, when the weather was cold. When head retraction was seen in a pigeon, the bird had usually lost from 70 to 90 g., though occasionally as little as 50 or as much as 110 g. Birds which did not show head retraction in 30 days were not used.

The pigeons were kept in the open air in cages with wire netting screens of $\frac{1}{2}$-inch diameter to prevent access to faeces. When a pigeon showed head retraction, it was taken to the laboratory, the dose was given by mouth, and without delay it was put again in the open air in a separate cage. Attention was not paid to symptoms other than those of typical head retraction. The pigeon was then observed at intervals and its condition recorded at the end of each period of 24 hours from the time the dose was given. The pigeon was observed for several days to see for how long it remained free from symptoms. If head retraction had fully disappeared at the end of 24 hours, but had reappeared by 48 hours, the result was recorded as a cure for 1 day. Freedom

from symptoms for 48 hours, but for less than 72 hours, was recorded as a cure for 2 days, and so on.

If a pigeon was not cured within a few hours of the administration of the dose, no further dose was given. All doses were given in about 5 cc. of water, half of the water being kept to wash down traces. A pipette fitted with a short rubber tube of 2 mm. internal diameter was used.

Preparations examined.

The preparations examined were (1) a sample of dried yeast supplied by the National Institute for Medical Research for an investigation undertaken as a preliminary to the International Conference in London, 1931; (2) a sample of activated acid clay prepared by Prof. Jansen of Amsterdam and supplied by the National Institute for Medical Research; (3) a second sample of acid clay proposed for use as the International Standard of the antineuritic vitamin, also supplied by the National Institute for Medical Research; (4) two concentrated solutions kindly given to us by Prof. Peters; (5) a commercial extract of rice polishings; (6) a commercial soft extract of yeast.

These six preparations appeared to us to provide a sufficiently diverse assortment of preparations of the antineuritic vitamin.

Experimental results.

It will be convenient to describe some results relating to the method of estimating potency used by Kinnersley *et al.* As already stated, the percentage of pigeons which we have observed to develop head retraction within 30 days when fed on polished rice is 45. Prof. Peters has kindly informed us that the percentage observed in his laboratory is almost the same. We suggest that the close correspondence between these percentages means that certain precautions taken in Prof. Peters's laboratory are unnecessary (*e.g.* feeding the pigeons on a uniform stock diet for several days before giving them the rice diet and washing the rice). The omission of these steps means a saving of time and expense in routine work.

The relation between dose and duration of cure.

We have examined the conclusion of Kinnersley *et al.* that there is a relation between the amount of the antineuritic factor supplied and the time of protection after cure, and the results are given in Table II.

The preparations examined were each administered in one or more doses, and the average duration of cure was calculated for each selected dose. Thus the average duration of the cure of 11 birds to which 0·03 g. of the original sample of acid clay was administered was 3·7 days; hence by Peters's method the potency was 123 day doses per g. In calculating the average duration of cure, no account was taken of birds to which the dose was administered and which were not cured. The final column in Table II gives the potency of each substance expressed as units per g. or per cc., taking the unit as the activity present in 0·01 g. of the standard acid clay. The different figures for the potency of each substance should of course be alike if the day-dose method of comparing the activity of the different preparations with the standard is satisfactory. Inspection shows that the different figures are by no means alike. The potency of the original sample of acid clay was found to be 357, 176, 104 and 91 units per g. To the first of these figures less attention can be paid since it is based

Table II. *Vitamin B_1 potency of various substances estimated in terms of the International Standard by day-dose method.*

Material	Dose	No. of birds cured	Average duration of cure (days)	Day doses per g. or cc.	Potency in terms of Standard units per g. or cc.
Acid clay (original sample)	0·015 g.	4	3·75	250	357
	0·03 g.	11	3·7	123	176
	0·06 g.	16	4·4	73	104
	0·12 g.	13	7·7	64	91
Acid clay (Standard)	0·03 g.	7	2·1	70	100
Dried yeast	0·031 g.	2	4·5	145	207
	0·062 g.	5	1·8	28·8	41
	0·125 g.	15	4·4	35·2	50
	0·25 g.	16	5·9	23·6	34
Vitamin B_1 solution (*a*)	0·1 cc.	4	2·0	20	29
(*b*)	0·2 cc.	6	1·8	9	13
(*a*)	0·25 cc.	7	2·1	8	11·4
(*b*)	0·35 cc.	9	2·5	7	10
Extract of rice polishings	0·25 cc.	5	1·6	6·4	9
	0·5 cc.	10	5·1	10·2	14
	1·0 cc.	10	5·7	5·7	8
	2·0 cc.	11	7·5	3·75	5·3
Yeast (soft extract)	0·12 g.	3	1·7	14	20
	0·24 g.	6	3·5	14	20

Note. The vitamin B_1 solutions (*a*) and (*b*) were two different tubes kindly supplied by Prof. Peters, which were probably, but not certainly, alike in potency. Since examination showed no difference between them, they have been tabulated as one preparation.

on a cure of only 4 birds; but there is no explanation of the difference between the estimate of 176 units per g. and the other estimates of 104 and 91 units per g., save that the error of the method is large, or that the relationship between duration of cure and dose of vitamin B_1 given is not a straight line. (See the section on the statistical examination of these results.) The potency of the extract of rice polishings was found to be 9, 14, 8 and 5·3 units per g., the highest value being nearly three times the lowest.

In spite of these divergences there are some similarities; the two values 104 and 91 for the original sample of acid clay are close together; the highest and lowest of the three figures for dried yeast, 41, 50 and 34 do not differ by more than 50 %; the three figures 13, 11·4 and 10 for the vitamin B_1 solution are close together; two of the figures for the extract of rice polishings are respectively 8 and 9, while the two figures for the soft extract of yeast are identical.

We draw the conclusion that there is certainly foundation for the view of Kinnersley *et al.* that a relation exists between the size of the dose of vitamin B_1 administered and the duration of the cure, but that, when groups of 10 or 15 birds are used for an estimation, errors of 50 or 100 % may occur. It will be observed that the potency in day-doses per g. or per cc. of the materials in Table II diminished as the dose which was examined grew larger; it follows that the potency of any unknown material expressed as day doses per g. will depend to some extent on the dose which is chosen, becoming lower as the dose becomes higher. It is right to point out that these comments apply only to experiments in which different pigeons are used for examining the effect of different doses. If the same group of pigeons had been used for testing the effect of each of the doses of the different substances mentioned in Table II, it is possible that we would have found that the average duration of cure was proportional to the

dose; Kinnersley *et al.* [1928] have published some evidence in favour of this expectation. For routine purposes, when a preparation is to be compared with the International Standard, the use of the same group of pigeons for both standard and unknown preparations would necessitate a long delay in the completion of a test.

The percentage of birds cured.

Apart from the attempt of Kinnersley *et al.* to estimate potency in terms of "day-doses," other workers have determined potency by finding the minimum curative dose. The difficulty of doing this arises from the variation in the response of different pigeons; head retraction is not cured in some birds by a dose of the vitamin which will satisfactorily cure others, and in practice it is impossible to define what is meant by a "minimum curative dose." It is tempting to explain differences of this kind by supposing that at the time head retraction occurs the deficient diet has left one bird less able to absorb the vitamin than another; but such explanations do not help to overcome the practical difficulty of estimation. Similar variations in the response of animals to drugs are well known in pharmacology, and Trevan [1927] showed how they were to be overcome. We have followed the principles demonstrated by Trevan in the following experiment.

The sample of acid clay first supplied by the National Institute for Medical Research and the sample of dried yeast were tested in different doses to determine the proportion of birds cured by each dose. Thus the dose of 0·015 g. acid clay was given to each of 16 pigeons with head retraction; 4 were cured and remained cured for at least 24 hours; the dose of 0·03 g. acid clay was given to each of another group of 16 birds, of which 8 were cured. The results are recorded in Table III. It will be seen that as the dose either of acid clay or of dried yeast was increased, the percentage of birds cured also increased. In

Table III. *Vitamin B_1 potency of various substances estimated in terms of the I.S. by the percentage of birds cured.*

Material	Dose	No. of birds given dose	No. cured	% cured	Units per g. or cc.	Weighted mean (units)
Acid clay (original sample)	0·015 g.	16	4	25	118	110
	0·03 g.	23	11	48	100	
	0·06 g.	20	16	80	115	
	0·12 g.	20	13	65	—	
Acid clay (Standard)	0·03 g.	15	7	47	100	100
Dried yeast	0·031 g.	16	2	12·5	32·1	33·8
	0·062 g.	16	5	31	35·7	
	0·125 g.	20	15	75	39·8	
	0·250 g.	20	16	80	27·7	
Vitamin B_1 solution (*a*)	0·1 cc.	13	4	31	22·1	16·2
(*b*)	0·2 cc.	10	6	60	17·8	
(*a*)	0·25 cc.	13	7	54	13·1	
(*b*)	0·35 cc.	13	9	70	12·4	
Extract of rice polishings	0·25 cc.	8	5	62	14·8	17·5
	0·5 cc.	12	10	83	24·6	
	1·0 cc.	12	10	83	12·3	
	2·0 cc.	12	11	92	—	
Yeast (soft extract)	0·12 g.	10	3	30	17·9	19·6
	0·24 g.	8	6	75	20·7	

Fig. 1 the percentage of birds cured has been plotted against the dose, and the points for the acid clay and for the dried yeast fit curves of the usual sigmoid shape. The point indicating the result of administering the largest dose of acid clay lies off the curve through the other points; it was observed that all the birds which were not cured by this dose were dead on the day after the dose was given. This unusual occurrence gave us the impression that the curative action of the large dose was complicated by a toxic effect.

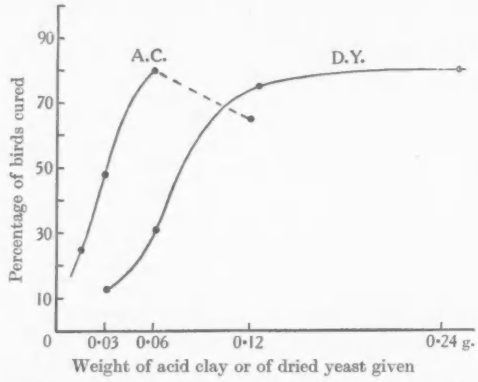

Fig. 1. A.C., Curve of response of polyneuritic pigeons to graded doses of activated acid clay (Jansen's preparation of vitamin B_1). D.Y., Curve of response of polyneuritic pigeons to graded doses of dried yeast.

From the two curves shown in Fig. 1 it is possible to make a quantitative comparison between the potency of the dried yeast and that of the acid clay. Thus a line parallel with the abscissa may be drawn to cut the curves at different points, and the ratio of the abscissae determined. Thus such a line drawn through the ordinate corresponding to 30 % cuts the acid clay curve at a point of which the abscissa is 0·02, and cuts the dried yeast curve at a point of which the abscissa is 0·06. The ratio of the potency of dried yeast to acid clay is, therefore, 0·02/0·06, or 0·33. Similarly the ratio of the potency determined by a line drawn through the ordinate corresponding to 50 % is 0·39, and that determined by a line drawn at 70 % is 0·42. The three figures 0·33, 0·39 and 0·42 give an average of 0·38.

The estimation of unknown preparations.

While it is true that a comparison of the potency of dried yeast with that of acid clay has been obtained by the foregoing experiments, it may be argued that the number of pigeons needed to construct a curve for an unknown preparation like the curve in Fig. 1 is too great for the method to be of practical value. The testing of unknown preparations can be abbreviated, however, in the following way. We have constructed from the two curves in Fig. 1 a third curve shown in Fig. 2, which is an approximation to the characteristic curve [see Trevan, 1927] for the response of pigeons with head retraction to doses of the antineuritic vitamin. The ordinates in Fig. 2 are, as in Fig. 1, the percentage of pigeons cured 24 hours after administration of the dose. The abscissae are doses expressed in arbitrary figures. The abscissa corresponding to the cure of

50 % of birds is given the value 1·0. Half-way between this point and the origin is 0·5, *etc.* The points on the acid clay curve in Fig. 1 are points corresponding to the cure of 25, 50 and 80 % of pigeons; if the dose curing 50 % be taken as 1·0, then the abscissae of these points in Fig. 2 are 0·5, 1·0 and 2·0, for the doses were in this proportion. Similarly the points on the dried yeast curve are plotted in Fig. 2 again taking the dose (determined by interpolation in

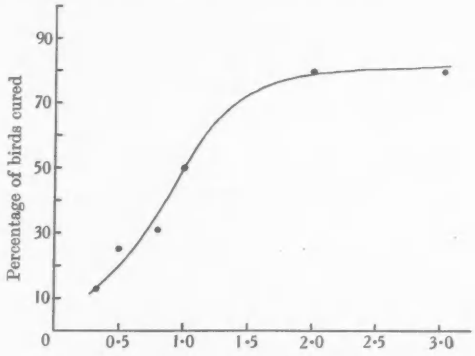

Fig. 2. Curve of response constructed from results of tests on acid clay and on dried yeast.

Fig. 1) which cures 50 % of pigeons as 1·0. The points on the two curves in Fig. 1, adjusted in this way, fit one curve remarkably well, the coincidence supporting the view that the same phenomenon is responsible for the shape of both curves in Fig. 1.

The curve in Fig. 2 can now be used for the estimation of unknown preparations of the vitamin, and the manner of its use is most simply explained by a comparison which was made of the two water clear solutions containing vitamin B_1, prepared by Prof. Peters, with the International Standard acid clay.

Comparison of vitamin B_1 solution with the Standard.

The vitamin B_1 solution was administered in a dose of 0·1 cc. to each of 13 birds suffering from head retraction; 4 birds were cured (approx. 30 %). The International Standard acid clay was administered to 15 birds suffering from head retraction in a dose of 0·03 g.; 7 birds were cured (approx. 50 %). The relative potency of doses curing 30 and 50 % of birds is given by Fig. 2 as 0·65 to 1·0.

Hence $$\frac{\text{0·1 cc. vitamin B}_1\text{ solution}}{\text{0·03 g. Standard}} = \frac{0{\cdot}65}{1{\cdot}0}$$

or $$\text{1 cc. vitamin B}_1\text{ solution} = \text{0·2 g. Standard}$$
$$= \text{20 units.}$$

A further examination of the vitamin B_1 solution was made in which 0·25 cc. was administered to each of 13 birds, of which 7 were cured, or approximately 50 %; hence 0·25 cc. vitamin B_1 solution was found approximately equivalent to 0·03 g. Standard, which contains by definition 3 units; hence 1 cc. = 12 units.

No further observations were possible with this material, as very little was left, but Prof. Peters kindly sent us another tube of solution which he believed to be similar to the first tube, though he was not certain. The small remainder of the first tube was mixed with this second tube, and the mixture was administered to pigeons in the two doses 0·2 and 0·35 cc. The first dose cured 6 out of 10 birds, indicating a potency of 17·5 units per cc., and the second dose cured 9 out of 13 birds, indicating a potency of 12 units per cc.

Comparison of the day-dose and percentage-cure methods.

The results obtained with the two solutions supplied by Prof. Peters made possible a useful comparison shown in Table IV of the day-dose method of calculating potency and the percentage-cure method. For this comparison the

Table IV. *Examination of vitamin B_1 solution.*

First tube, A; second tube, B.

Dose (cc.)	Units per cc. Day-dose method	Average	Percentage-cure method	Average
0·1 A	29	15·9	20	15·4
0·2 B	13		17·5	
0·25 A	11·6		12	
0·35 B	10		12	

two solutions examined were assumed to be identical in potency; the results indicated that they were. The results by the day-dose method have already been given in Table II.

Observations on other preparations.

The extract of rice polishings and the soft extract of yeast were also examined by the percentage-cure method. In a dose of 0·25 cc. the extract of rice polishings cured 5 out of 8 pigeons; in a dose of 0·12 g. the soft extract of yeast cured 3 out of 10 pigeons, while a dose of 0·24 g. cured 6 out of 8 pigeons. From these results the potency in units per cc. or per g. has been calculated and expressed in Table V.

Table V. *Comparison between results obtained by the day-dose and percentage-cure methods.*

Material	Dose	Potency by day-dose method (units per g. or cc.)		Potency by percentage-cure method (units per g. or cc.)	
Acid clay (original sample)	0·03 g.	176	123	100	
	0·06 g.	104			
	0·12 g.	91			
Dried yeast	0·062 g.	41	42	34	
	0·125 g.	50			
	0·25 g.	34			
Extract of rice polishings	0·25 cc.	10·8		12	
Yeast (soft extract)	0·12 g.	20	20·5	17	18·5
	0·24 g.	21		20	

The agreement seen in Table V between the average figures for the potency by the day-dose method and for the potency by the percentage-cure method is striking; for the original sample of acid clay the agreement would be close

but for the one high estimation by the day-dose method. The average figures for the two methods differ by 20 % for dried yeast, by 11 % for the extract of rice polishings, while the means of the two pairs of results for the soft extract of yeast differ by 10 %. The two methods have given the same average results in these experiments because several doses were tested, and the high values calculated from the low doses in the day-dose method balanced the low values calculated from the high doses.

Non-specific cures.

The pigeon curative method has been criticised because some other substances than the antineuritic vitamin will give temporary relief from head retraction in some birds.

Two questions appear to us to be confused in the minds of those who offer this criticism, namely whether a substance contains any vitamin B_1, and how much vitamin B_1 it contains. In detecting the presence of any vitamin B_1, it is obviously important to watch for non-specific effects; on the other hand, in determining the potency of a substance known to contain vitamin B_1, it is less important, since the substances which produce non-specific cures can usually be assumed to be absent. Histamine, pilocarpine, nitrites, thyroxine and choline [Dutcher, 1919; Abderhalden, 1923; Peters, 1924] are not present in extracts of yeast or rice polishings in sufficient quantity to interfere with the estimation of the vitamin B_1.

Peters adopts the precaution of administering dextrose to each bird which develops head retraction; we have, therefore, examined the effect of giving 0·05 g. dextrose in 5 cc. water to a series of pigeons prepared here. Out of 16 birds to which the dose was administered, 2 were cured for periods of 24 and 48 hours respectively. It follows from this result, that if we were examining a substance of unknown origin, in which dextrose might be present, it would be wrong to calculate the amount of vitamin from the cure of only a small proportion of birds; it would be necessary to test the effect of a larger dose. It is of course wise to do this whatever the origin of the preparation being tested; thus, in testing the soft extract of yeast as already described, the potency was first calculated from the cure of 3 birds out of 10 by a dose of 0·12 g.; a further estimate was then made by administering twice the dose, when 6 out of 8 birds were cured. The final estimate of 18·2 units per g. did not differ greatly from the first estimate of 16·6 units per g.

Birds cured for 1 day.

The definition of a cure of head retraction given earlier in the paper was that a bird should be found free from symptoms at the end of 24 hours after the dose was administered. If a bird found to be cured at that time developed symptoms again 48 hours after the first sign of head retraction, the cure was counted as a cure for 1 day. The opinion was expressed to us that it was unsafe to assume that a cure for less than 48 hours was a genuine cure. We have, therefore, recalculated our results, excluding all birds in which the cure lasted for less than 48 hours. We have found, however, that the results so obtained did not differ appreciably from those already given in Tables II and III. Thus the potency of dried yeast became 47 units per g. instead of 42 units per g. when using the day-dose method of comparison and became 34 units per g. instead of 38 units per g. using the percentage-cure method.

Duration of head retraction before dosing.

When pigeons developed head retraction on the diet of rice, some were observed at the beginning of the day, and some were observed later; as no inspections were made during the night, birds found with head retraction in the morning may have had the symptoms for 15 hours before the dose was given, whereas birds found later in the day were given the dose within an hour or two of the onset. We have examined the records to see whether the birds in a group which were not cured by a particular dose were those which were found with symptoms in the early morning, and whether the birds which were cured were those found later. The examination showed no such correlation; the cure of head retraction by a particular dose was not dependent on the lapse of a short time between the onset of symptoms and the administration.

PART II. RATS.

The growth-promoting property of vitamin B_1.

It has been shown by many workers that vitamin B_1 (as distinct from vitamin B_2 or the vitamin B complex) is necessary for the growth of the rat. Sherman and Axtmayer [1927] demonstrated the supplementary nature of wheat germ and dried milk and concluded that this was due to the greater amount of vitamin B_1 in wheat germ than in dried milk. Chick and Roscoe [1927] found that an extract of the antineuritic vitamin prepared from yeast according to Peters's method would not promote growth in rats fed on a diet deficient in vitamin B (complex). If, however, this was supplemented by a daily dose of yeast autoclaved at 120° for 5 hours to destroy the antineuritic vitamin, growth was resumed. They showed also that rats lose weight and die more quickly on a shortage of vitamin B_1 than on a shortage of vitamin B_2. Hassan and Drummond [1927] showed that two factors in yeast, differentiated by their behaviour to alkalis, were necessary for the normal rate of growth of rats fed on high-protein diets. It then became generally recognised that, in order to estimate vitamin B_1 by means of its growth-promoting property, vitamin B_2 must be liberally supplied to the rats.

Aykroyd and Roscoe [1929], in estimations of vitamin B_2, gave a daily dose of 0·1 cc. of Peters's antineuritic concentrate (equivalent to 0·6 g. yeast) to their rats in addition to the vitamin B-free diet.

Finally, Chick and Roscoe [1929] developed a method for the estimation of vitamin B_1 in foodstuffs by means of its growth-promoting property. They supplied vitamin B_2 by a daily dose of autoclaved yeast (120° for 5 hours) or substituted the purified caseinogen of the diet by coagulated egg-white from fresh eggs, which they had shown to contain vitamin B_2. They discarded the latter method, however, as the growth of the rats was not maintained long enough for the test, and they concluded that autoclaved yeast contains a heat-stable factor necessary for growth and distinct from both vitamin B_1 and vitamin B_2. Fresh egg-white does not contain this factor.

Roscoe [1930; 1931, 1, 2] compared the vitamin B_1 content of various vegetables and fruits by determining the dry weight of each necessary to produce a given rate of growth in 5 weeks. Vitamin B_2 was given to each rat as a daily dose (equivalent to 0·5 g. dry yeast) of an extract made from washed brewer's yeast by boiling it with 0·01 % acetic acid and then autoclaving the extract for 5 hours at 120° (p_H *ca.* 5·0).

Halliday [1932] supplied vitamin B_2 as 15 % autoclaved yeast in the basal diet of rats which were used for estimations of vitamin B_1.

Guerrant and Dutcher [1932] used as a source of vitamin B_2 a dry autoclaved (6 hours, 15 lbs.) extract of yeast which had been precipitated at an 80 % alcohol concentration of the aqueous extract in the filtrate from 50 % alcohol concentration.

Chick and Jackson [1932], in estimating vitamin B_1 with the rat as the experimental animal, used as a source of B_2 a small daily ration of a watery yeast extract, autoclaved for 4 hours at 120° at p_H 5·0.

It then seemed to us that it would be useful to construct a curve of response relating average increase in weight of groups of rats to daily dose of vitamin B_1 given, as we had previously done for vitamin A and vitamin D.

I. *Diet of the rats.*

Young rats weighing 55–70 g. were given a diet consisting of:

	%
Caseinogen (light white B.D.H. not extracted) ...	15
Dextrinised rice starch	71
Agar-agar	2
Salt mixture (Steenbock's 40)	4
Autoclaved yeast...	8

Each rat was given 5 drops of a good sample of cod-liver oil twice a week to supply vitamins A and D.

The yeast was spread on tins in thin layers, 40 g. over 44 sq. in., and autoclaved for 6 hours at 15 lbs. additional pressure. It became dark brown in colour and moist. It was dried by leaving overnight in an electric oven whose current had been turned on until the temperature was raised to 100° and then turned off before the trays of yeast were put into it. The yeast was caked after drying and had to be ground in a mortar.

The rats were given this diet until they ceased to grow. During this preparatory period of 14 to 18 days, nearly every rat increased in weight by at least 10 g., many by 20 g. and some even by 30 g. Those which were given no vitamin B_1 after growth ceased, lost weight rapidly and died in 21–38 days from the beginning of the preparatory period.

The rats were kept 4 together in a cage during the preparatory period, on grids of 0·3 in. mesh. During the giving of doses, each rat was kept in a separate cage on a grid of 0·5 in. mesh.

Twelve litters of 5 or 6 rats each were used for testing 5–6 doses of acid clay, each rat of a litter receiving a different dose of acid clay. The doses tested were 0, 0·005, 0·01, 0·02, 0·04, 0·1 g. For the first 6 litters each dose was weighed daily and mixed with a little water in a separate dish for each rat. Some of the rats refused to eat their doses completely but when the dose was mixed with a little dextrin it was always eaten. For the later work, the acid clay was mixed at once with dextrin (1 part acid clay to 4 or to 9 parts dextrin as convenient) and the diluted material used for all the rats.

II. *First curve of response to graded doses of activated acid clay, the International Standard of reference for vitamin B_1 (8 % autoclaved yeast in the diet).*

No rats developed polyneuritis or convulsions [Smith, 1930] during this experiment, whether they received a dose of acid clay or not.

The growth response of the rats was graded to the dose of acid clay given. The male rats made greater increases in weight on each dose (but one) than

the female rats (Fig. 3), but the difference was less than the difference in similar experiments with vitamins A and D [Coward *et al.*, 1931; 1932]. The mean increases in weight of the bucks and does respectively have themselves been averaged for constructing a curve of response for use in interpreting estimations on other substances. The results of the tests on other substances have been treated similarly for the sake of simplicity.

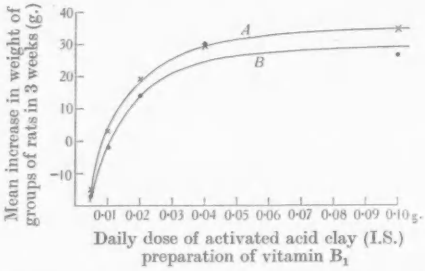

Fig. 3. Curves of response of groups of rats to different doses of activated acid clay (I.S. for vitamin B_1 estimations). Each rat of a group received the same daily dose for 3 weeks. The basal diet contained only 8 % autoclaved yeast as a source of vitamin B_2.

A, determinations from male rats. *B*, determinations from female rats.

In the first 5 litters we used for the construction of this curve, several of the rats given the highest dose of acid clay (0·04 g.) grew less quickly than was expected from the growth of the rats on the lower doses. We, therefore, used one rat of each of the later litters for testing a dose of 0·1 g. acid clay. The response of the bucks to this dose was higher than to the dose of 0·04 g. but that of the does was slightly less. This, together with results of tests of other substances made at the same time, led us to the conclusion that the basal diet we were using was deficient in some substance or substances other than vitamin B_1. We suspected the destruction of vitamin B_4 in the autoclaving of the dried yeast. We were able to examine this possibility through the kindness of Dr V. Walker (Reader) who gave us sufficient solution of vitamin B_4 to dose 12 of our rats receiving different doses of acid clay, but growing very slowly. This did not, however, increase the rate of growth of any one of our rats, and we concluded that the diet was not lacking in vitamin B_4. We then suspected a deficiency of vitamin B_2 or some other heat-labile factor in the basal diet. Tests on other substances (Section III) and the construction of a fresh curve from rats given a basal diet containing 20 % autoclaved yeast (Section IV) confirmed this view.

III. *Comparison of other substances with activated acid clay with regard to their vitamin B_1 potency.*

Four substances were examined for their content of vitamin B_1 at the same time that the curve of response to doses of activated acid clay was being constructed. The substances were (*a*) a sample of dried yeast (used by several laboratories in connection with preliminary work on the International Standard), (*b*) a sample of wheat embryo (also used by the same laboratories for the same work), (*c*) a commercial sample of a food and (*d*) a commercial sample

of a yeast extract (I). Each was tested in two different doses. It happened that the lower dose of each substance gave a result comparable with the result on a low dose of acid clay, but the higher dose of each substance gave a result considerably higher than that given by the highest dose of acid clay. The mean increase in weight produced by each of the higher doses was well above the curve. In Fig. 4 the mean increase in weight of the animals given the lower

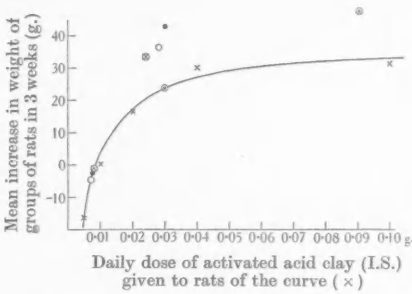
Mean increase in weight of groups of rats in 3 weeks (g.)
40
30
20
10
0
-10
0·01 0·02 0·03 0·04 0·05 0·06 0·07 0·08 0·09 0·10 g.
Daily dose of activated acid clay (I.S.) given to rats of the curve (×)

Fig. 4. Uselessness of this curve of response to estimate the vitamin B_1 content of other substances. (Each point is the average of the mean increases in weight of the bucks and does respectively, *i.e.* no point is overweighted by an excess of results from bucks.)

× = Mean increases in weight of the groups of rats given graded doses of the Standard.
▪ = Mean increases in weight of groups of rats given daily 0·05 and 0·2 g. respectively of a sample of dried yeast I.
⊙ = Mean increases in weight of groups of rats given daily 1·0 and 3·0 g. respectively of a commercial food sample.
⊗ = Mean increases in weight of groups of rats given daily 0·1 and 0·3 g. respectively of a sample of wheat embryo.
○ = Mean increases in weight of groups of rats given daily 0·05 and 0·2 g. respectively of yeast extract I.

The result from the lower dose of each test was plotted on the curve itself. The result from the higher dose of each test was plotted against the abscissa corresponding to the appropriate multiple of the abscissa found for the lower dose.

dose of the substance was marked on the curve, the abscissa of that point noted, and the mean increase in weight of the animals given the higher dose was plotted against the abscissa which bore the same ratio to the first abscissa that the higher dose bore to the lower dose of substance tested. It was obvious that the dried yeast, the wheat embryo, the yeast extract and the food substance each contained some substance necessary for growth which was not supplied in sufficient amounts by our basal diet *plus* large doses of acid clay.

We, therefore, decided to repeat the experiment using 20 % autoclaved yeast in the diet which was then made up as follows.

	Parts
Caseinogen (light white, B.D.H. untreated)	15
Dextrinised rice starch	79
Agar-agar	2
Salt mixture (Steenbock's 40)	4
Autoclaved yeast...	25

The same amount of cod-liver oil was given to each rat as before.

IV. *Second curve of response relating mean increase in weight of groups of rats to dose of vitamin B_1 given (20 % autoclaved yeast in basal diet).*

A second curve of response was constructed by the same method as the first, but 20 % yeast autoclaved under apparently the same conditions was used instead of 8 %. Four litters of rats were used for this experiment. The curve of response was steeper than the first (Fig. 5). It justified our conclusion that in the former experiment we had not given a large enough percentage of autoclaved yeast in the basal diet.

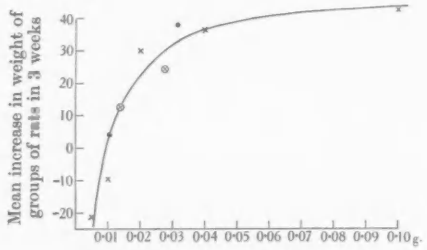
Mean increase in weight of groups of rats in 3 weeks
40
30
20
10
0
-10
-20
0·01 0·02 0·03 0·04 0·05 0·06 0·07 0·08 0·09 0·10 g.
Daily dose of activated acid clay (I.S.) given to the rats of the curve (×)

Fig. 5. Curve of response of groups of rats to graded doses of activated acid clay (I.S. for vitamin B_1 estimations). Each rat of a group received the same daily dose for 3 weeks. The basal diet contained 20 % autoclaved yeast as a source of vitamin B_2.

Usefulness of this curve for estimating the vitamin B_1 content of other substances.

× = Mean increases in weight of groups of rats given graded doses of the Standard.

• = Mean increases in weight of groups of rats given daily 0·05 and 0·15 g. respectively of dried yeast.

⊗ = Mean increases in weight of groups of rats given daily 0·1 and 0·2 g. respectively of a yeast extract II.

The results were plotted as in Fig. 4.

V. *Confirmation of the validity of the second curve of response relating mean increase in weight of groups of rats to dose of vitamin B_1 given.*

Two substances, dried yeast II and a yeast extract II (both different from those used previously), were tested in 2 doses each. The mean increases in weight of the 2 groups of rats given the lower doses of the 2 substances were plotted on the curve, and the mean increases in weight of the rats given the higher doses were plotted against the abscissae which bore the same ratios to the abscissae of the lower doses as the ratios of the actual doses. These points fell very nearly on the curve of response, one somewhat above the curve, the other somewhat below (Fig. 5). Thus the second curve appears to be a better one than the first in that, so far as it has been tested, the responses do not seem to be limited by an insufficiency of any factor in the basal diet. The response to a test substance when this diet is used appears to be a response to vitamin B_1 only.

VI. *The possible logarithmic nature of the curves.*

Each of the two curves described in this paper was constructed from 5 determinations of the mean increases in weight in 3 weeks of 5 groups of rats, the rats of each group being given daily doses of 0·005, 0·01, 0·02, 0·04 and 0·1 g. International Standard preparation of vitamin B_1. The curves were drawn as smoothly as possible near the points plotted on graph paper. Neither of the

curves so drawn was logarithmic, and the best logarithmic curve through either set of 5 points (determined from the best straight line through the points obtained by plotting mean increases against the logs of the doses) did not fit the actual points at all well. But a logarithmic curve fitted the four lower points of the first set very well and another fitted those of the second set only rather less well (Fig. 6). This is similar to our finding in our vitamin A work, in which

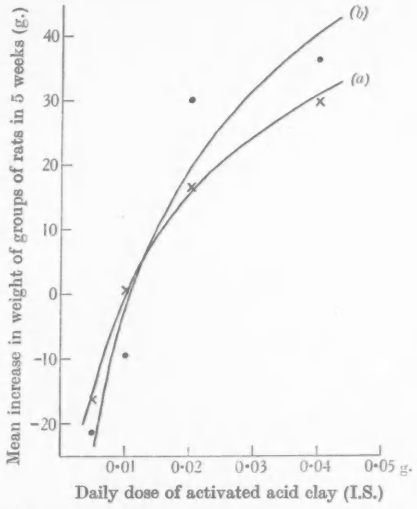

Fig. 6. The best logarithmic curves relating the mean increase in weight of groups of rats to dose of vitamin B_1 given when the basal diet contains (*a*) 8 % and (*b*) 20 % respectively of autoclaved yeast as a source of vitamin B_2.

a logarithmic curve fitted the responses of rats (mean increases in weight in 5 weeks) to doses of 0·25, 1·0, 1·5, 2·5, 7·5 mg. respectively of cod-liver oil, but not the response to the highest dose, 20·0 mg. It should, however, be safe to accept the logarithmic equations for the curves for the range of mean increases which determined the shapes of the curves.

The equation for the first vitamin B_1 curve reported in this paper (Fig. 6 (*a*)) for the range -16 to $+30$ g. mean increase in weight is $y = 102{\cdot}3 + 51{\cdot}1 \log x$.

The equation for the second curve reported in this paper (Fig. 6 (*b*)) for the range -21 to $+36$ g. mean increase in weight is $y = 139{\cdot}4 + 70{\cdot}4 \log x$.

VII. *Justification for the acceptance of the logarithmic equations for the curves of response.*

The logarithmic curve through the first 4 points in the first experiment fits those points so well that it can be accepted without question as representing the relation between response and dose of vitamin B_1 given for the range of doses 0·005 to 0·04 g. I.S. Its equation is $y = 102{\cdot}3 + 51{\cdot}1 \log x$.

The logarithmic curve through the first 4 points in the second experiment fits those points less well and it might be accepted with some hesitation. When, however, the results of feeding 2 different doses of each of 2 different substances were interpreted by means of this curve, the ratios of the apparent potencies

of the respective pairs of doses were so nearly the known ratios of the doses that we feel no hesitation in accepting the curve with its logarithmic equation as representing the relation between the mean increase in weight of a group of rats (all given the same dose) and the dose of vitamin B_1 given (Table VI). Its equation is $y = 139{\cdot}4 + 70{\cdot}4 \log x$.

Table VI. *Confirmation of validity of second curve of response relating mean increase in weight in 3 weeks of groups of rats to dose of vitamin B_1 given (I.S.).*

Substance tested	Daily dose (g.)	No. of rats	Mean increase in wt. in 3 weeks (g.)	Abscissa of curve corresponding to the increase in weight	Ratio of potencies of doses according to ratio of abscissae	Actual ratio of doses
Dried yeast II	0·05	3	4·2	0·0125	1 : 2·9	1 : 3
,,	0·15	4	38·0	0·0365		
Yeast extract II	0·1	4	12·8	0·0160	1 : 1·6	1 : 2
,,	0·2	4	24·5	0·0255		

VIII. *Interpretation, by means of the second curve, of results which could not be interpreted by means of the first curve.*

In Table VII are collected details of the tests of the four substances mentioned in Section III, which could not be interpreted by means of the curve of

Table VII. *Interpretation, by means of the second curve, of results which could not be interpreted by means of the first curve.*

Substance tested	Dose (g.)	No. of rats ♂	No. of rats ♀	Mean increase in wt. in 3 weeks (g.) ♂	Mean increase in wt. in 3 weeks (g.) ♀	Average mean increase of ♂ and ♀ rats	Interpretation by second curve of response. Diet contained 20 % autoclaved yeast: Abscissa corresponding to mean increase in weight	Apparent ratio of doses	Actual ratio of doses
Dried yeast I	0·05	6	4	−1·3	−4	−2·65	0·0096	1 : 4·2	1 : 4
	0·2	7	3	49·7	36·0	42·85	0·0407		
Food substance	1·0	3	3	23·3	23·7	23·5	0·0226	1 : 2·1	1 : 3
	3·0	3	3	55·3	37·6	46·45	0·0467		
Wheat embryo	0·1	4	2	0·75	−3·0	−1·12	0·0101	1 : 3·5	1 : 3
	0·3	2	4	39·5	27·25	33·37	0·0352		
Yeast extract I	0·05	3	2	−6·7	−2·5	−4·6	0·0090	1 : 3·8	1 : 4
	0·2	3	2	39·3	33·0	36·15	0·0341		

response constructed about the same time and with the use of the same basal diet (containing 8 % autoclaved yeast). When these are interpreted by means of the second curve of response (basal diet containing 20 % autoclaved yeast), the ratios of the apparent vitamin B_1 potencies of the respective pairs of doses are found to be in very good agreement with the known ratios of the doses given.

This may be taken as good evidence that the basal diet which contained only 8 % autoclaved yeast was deficient in some factor necessary for growth which was not supplied by the activated acid clay but which was supplied by each of the other substances examined.

The potency of all substances examined has, therefore, been calculated by means of the second curve and stated in terms of the I.S. for vitamin B_1 in Table VIII.

Table VIII. *Potency of substances tested by the rat method in terms of the I.S. for vitamin B_1.*

Substance tested	Dose (g.)	Abscissa corresponding to mean increase in weight	No. of units vitamin B_1 per g.
Dried yeast I	0·05	0·0096	19·2 } Average 19·8
	0·2	0·0407	20·35 }
Food substance	1·0	0·0226	2·26 } " 1·9
	3·0	0·0467	1·56 }
Wheat embryo	0·1	0·0101	10·01 } " 10·9
	0·3	0·0352	11·73 }
Yeast extract I	0·05	0·0090	18·0 } " 17·6
	0·2	0·0341	17·05 }
Dried yeast II	0·05	0·0125	25·0 } " 24·7
	0·15	0·0365	24·4 }
Yeast extract II	0·01	0·0160	16·0 } " 14·4
	0·2	0·0255	12·8 }

Discussion.

The point that appears to us of the greatest interest in this examination of the growth response of rats to graded doses of vitamin B_1 is that the slopes of the two curves of response obtained with 8 and 20 % respectively of autoclaved yeast in the basal diet are different. One might have expected the two curves to be superposed at their lower ends and the curve obtained with 8 % autoclaved yeast to bend away suddenly from the other one at the point where the maximum effect allowed by 8 % autoclaved yeast was reached. This, however, is not what happens. There is no sudden limiting of the response to vitamin B_1 when the supply of vitamin B_2 is insufficient. The inadequacy of the supply of vitamin B_2 affects the response to lower doses of vitamin B_1 as well as to higher doses. Thus, with only 8 % autoclaved yeast in the basal diet, a dose of some substance containing vitamin B_2 as well as vitamin B_1 would give a higher result than the same amount of vitamin B_1 in a substance free from vitamin B_2; provided the results fell on the curve, no error would be suspected (*cf.* our results depicted in Fig. 4) and the estimation of vitamin B_1 would be too high. This is not only of practical importance, but it seems to be of theoretical importance also as indicating an interdependence of the vitamins.

The difference in slope of the curves seems to indicate that the more vitamin B_2 (or some other factor, not B_2 or B_4, in the autoclaved yeast) in the basal diet, the greater is the response of rats to doses of vitamin B_1. Below the point of intersection (5 g. mean increase, 0·0125 g. Standard), the reverse appears to be true.

This is a similar result to the one reported by us [Coward *et al.*, 1932] concerning the dependence of the response to vitamin D on the amount of vitamin A in the basal diet (or given daily). We found that the larger the dose of carotene given to all rats daily, the greater was the slope of the curve of response to graded doses of vitamin D.

We do not conclude from these experiments that 20 % yeast autoclaved at 120° for 5 hours will always provide sufficient of the B vitamins (other than B_1)

to support normal growth. Indeed we have evidence (to be reported in a later paper) that 20 % of yeast autoclaved apparently in the same way may be far too small an amount for normal growth. We consider that our results demonstrate afresh the need for making a simultaneous test on at least one dose and preferably on two doses of a standard of reference whenever an examination of the vitamin B_1 content of a substance is made. The test can only be considered satisfactory if there is no evidence (such as we described in Section III) that the substance under test is supplying some factor other than vitamin B_1 which is not contained in sufficient amount in the basal diet.

PART III. THE VITAMIN B_1 POTENCY OF A SUBSTANCE AS ESTIMATED BY MEANS OF (*a*) PIGEONS AND (*b*) RATS.

The decision whether the tests by means of pigeons and the tests by means of rats measure the same factor rests on the results obtained from the examination of substances by both the methods. If both methods give the same result it may be assumed that the two methods measure the same factor. Two substances have been examined by both methods: (*a*) a soft extract of yeast I and (*b*) a sample of dried yeast I (Table IX).

Table IX.

	Potency of substances as determined by tests on		
	(*a*) pigeons		(*b*) rats
Substance tested	as calculated by day-dose method (units)	as calculated from % birds cured (units)	as calculated from mean increase in weight in 3 weeks (units)
Soft extract of yeast I	20·5	18·5	17·6
Dried yeast I	42·0	34·0	19·8
Dried yeast III	113·0	83·0	37·8

There is good agreement between the results of the tests of the soft yeast extract obtained by all three methods, but the pigeon test of the dried yeast makes it appear to be twice as potent as does the rat test. The chance that this divergence is due to the inaccuracy which is inevitable in all three tests has been calculated as about 1 in 4000, which is exceedingly small. It appears therefore, that for dried yeast, pigeons give a higher value for the vitamin B_1 potency than do rats. Confirmation of this result has recently been obtained by the examination of a third sample of dried yeast (III, Table IX)[1]. The day-dose method gives results which vary according to the size of dose given as shown in Parts I and IV of this paper, therefore very little attention need be given to the result obtained by this method of calculating the potency of the dried yeast. The result from the percentage of birds cured, however, confirms the result obtained with the first sample of dried yeast; *viz.* the pigeon method of calculating the vitamin B_1 potency of dried yeast gives a higher figure than the rat method. Actually the potency obtained by the pigeon method is about double of that obtained by the rat method.

[1] This test was made in the course of a comparison of different workers' methods of estimation of vitamin B_1 arranged by Prof. A. Jung, University of Basel.

It appears, therefore, that there is some reason for thinking that the rat is less able to respond to the vitamin B_1 contained in dried yeast than is the pigeon. On the other hand, the rat gave the same result as did the pigeon for the soft extract of yeast I, and from this it must be concluded that the rat and pigeon can respond equally well to the vitamin B_1 in such an extract and also to that in the acid clay. Thus the rat-growth method may prove to be unsuitable for estimating the B_1 potency of some substances, but, on the other hand, polyneuritic pigeons are obviously not suitable for estimating the vitamin B_1 potency of bulky substances such as wheat germ or wholemeal bread. The pigeon test moreover has a probable error of $+31$ or -26 % when 9 birds are used for a single dose of a substance, whereas the rat test has a probable error of only $+6{\cdot}1$ to $-5{\cdot}5$ % when 9 animals are used. It must be recognised, however, that the pigeon test is one in which the estimation is based on a specific reaction to vitamin B_1 and it must, therefore, on general principles, be preferable to a growth test.

PART IV. FURTHER CONCLUSIONS DRAWN FROM A STATISTICAL EXAMINATION OF THE RESULTS.

1. Pigeons.

A. Method of calculating results by duration of cure.

(a) Standard deviation of the average duration of cure.

The mean variance of the number of days of the pigeons' cure was calculated from the 19 tests recorded in Table II by the formula $\sigma^2 = \frac{\Sigma d^2}{N-m}$ where N = the number of observations (*i.e.* the number of pigeons) and m = the number of means (*i.e.* the number of tests). It was found that

$$\sigma^2 = 9{\cdot}02,$$

whence

$$\sigma = 3.$$

The number of pigeons from which the calculation was made is admittedly small, but it is large enough to use the value of σ found as a basis for certain calculations.

(1) *The standard deviation of the duration of the cure in relation to the duration of the cure.* It was to be doubted whether the variation in length of cure would be the same for pigeons given small doses as for pigeons given large doses of vitamin B_1. The results were, therefore, divided into 3 groups according to the average number of days of cure of the pigeons of the groups. The mean variance for each group was calculated and from it the standard deviation (Table X).

Table X.

No. of pigeons in group	Average no. of days cured	Standard deviation of mean
46	2·0	1·2
54	4·1	3·03
60	6·4	3·75

Thus there seems to be some evidence that there is less variation in the results obtained with low doses of vitamin B_1 than in those obtained with high doses.

(2) *Comparison between solids and liquids with regard to the pigeon's power of extracting and absorbing vitamin B_1 from them.* It might be expected that a pigeon in an abnormal state of health would be less able to utilise the vitamin B_1 in a solid preparation such as dried yeast or even activated acid clay than that in a liquid preparation such as Peters's concentrate, the liquid extract of rice polishings or the soft extract of yeast which was diluted with water before being given to the bird. Calculations were, therefore, made of the mean variances found in the tests on the liquid and solid preparations respectively. The values of σ ($= \sqrt{\text{mean variance}}$) of the two groups were found to be 2·93 and 3·06. These cannot be regarded as different. As the average duration of a cure of all the pigeons given a liquid preparation was 3·96 and that of all the pigeons given a solid preparation was 4·60 days it is evident that the similarity in the values of σ for the two groups has not arisen through a difference in absorbability being balanced by a difference in the average duration of cures of the two groups. Thus it must be concluded that pigeons such as were used in these tests can absorb vitamin B_1 from liquids and from solids such as the I.S. and dried yeast equally well.

(*b*) *Relation of duration of cure to dose of vitamin B_1 given.*

A curve has been constructed relating the duration of cure to dose of vitamin B_1 given. The data obtained from the doses of acid clay alone were not considered sufficient for constructing a curve for general application. Therefore, the acid clay equivalent of each dose of substance tested was calculated from the average value of that substance stated in Table III. The results were then averaged in 6 groups according as the dose given was (*a*) 0·011 to 0·016, (*b*) 0·021 to 0·024, (*c*) 0·030 to 0·032, (*d*) 0·040 to 0·048, (*e*) 0·056 to 0·060, (*f*) 0·085 to 0·087, (*g*) 0·12 to 0·175, (*h*) 0·35 g. of acid clay or its equivalent, and plotted as a curve relating average duration of cure to dose of acid clay (I.S. for vitamin B_1) (Fig. 7, continuous line). This curve is not logarithmic.

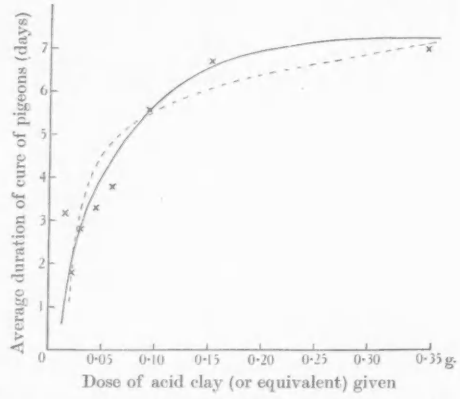

Fig. 7. Curve of response constructed from the duration of cures of polyneuritic pigeons given graded doses of activated acid clay or the equivalent of these doses in other substances.
Continuous line—best curve drawn through points obtained experimentally.
Broken line—best logarithmic curve through these points, but not adopted as it fits the points much less well than the other curve.

The best logarithmic curve through the points has been drawn as a broken line. It does not fit the points as well as the first curve drawn and there seems to be no reason for expecting the curve to be logarithmic; it has not been used.

(c) *The degree of accuracy obtainable by this method.*

The probable error of this test as obtained under the conditions described for this laboratory has been calculated in Table XI.

Table XI. *The probabilities of obtaining certain degrees of accuracy in this test. Standard error of duration of cure when 9 birds are used in a test.*

$$\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{3}{\sqrt{9}} = 1.$$

Average duration of cure, say,	Abscissa corresponding to duration of cure	Range of duration of cure	Range of abscissae* corresponding to range of cure	% range of abscissae
4 days	0·049	(*a*) $\pm\frac{2}{3}\epsilon = \pm 0{\cdot}67$ ($\frac{1}{2}$ chance)		
		4·67	0·064	130·6
		3·33	0·036	73·5
		(*b*) $\pm\epsilon = \pm 1{\cdot}0$ ($\frac{2}{3}$ chance)		
		5·0	0·074	151·0
		3·0	0·032	65·3
		(*c*) $\pm 2\epsilon = \pm 2{\cdot}0$ ($\frac{21}{22}$ chance)		
		6·0	0·110	224·5
		2·0	0·022	44·9

* Obtained by direct reading from curve (continuous line).

Thus the probable error of an estimation of vitamin B_1 by the use of a curve relating duration of cure to dose of vitamin B_1 given and using 9 pigeons for one dose of the substance under examination is about $+31$ or -26 %. We have not yet sufficient evidence to say whether a simultaneous test on the Standard should be made with every test of an unknown substance. On general principles it would seem to be desirable. The probable error of the estimation of the unknown in terms of the Standard then becomes $(+31 \text{ or } -26) \times \sqrt{2}$ which becomes $+43{\cdot}7$ or $-37{\cdot}0$ %.

It is obvious from the shape of this curve of response that, as pointed out in the early part of this paper, if a high dose of vitamin B_1 is given the result calculated as the day-dose response (*i.e.* days of cure/dose) will be lower than when a low dose of vitamin B_1 is given. The method of calculating the potency of a substance from days of cure/dose assumes a straight line relationship between duration of cure and dose given. The work in this paper shows that the relationship is curvilinear. Therefore, the day-dose method of calculating results can only be used over a very limited range of duration of cure. It resolves itself into a determination of the dose of the unknown substance required to give a cure of some chosen number of days and leaves no means of interpreting results which are of longer or shorter duration than the period chosen.

(d) *Confirmation of the curve relating duration of cure to dose of vitamin B_1 given.*

In the fifth column of Table XII are given the doses of acid clay corresponding to the mean duration of cure in each test. In the last column is stated in units per g. the potency of each substance as determined from each dose tested. The results from the different doses of any one substance are in good agreement

Table XII. *Vitamin B_1 potency of different substances in terms of the I.S., estimated by duration of cure method.*

Material	Dose	No. of birds cured	Average duration of cure (days)	Equivalent dose of acid clay (g.)	No. of units per g.
Acid clay (original sample)	0·015 g.	4	3·75	0·0435	290
	0·03 g.	11	3·7	0·0425	142
	0·06 g.	16	4·4	0·058	97
	0·12 g.	13	7·7	0·320	267
Acid clay (Standard)	0·03 g.	7	2·1	0·022	73
Dried yeast	0·031 g.	2	4·5	0·0605	195
	0·062 g.	5	1·8	0·020	32
	0·125 g.	15	4·4	0·058	43
	0·25 g.	16	5·9	0·1055	42
Vitamin B_1 solution (*a*)	0·1 cc.	4	2·0	0·021	21
(*b*)	0·2 cc.	6	1·8	0·020	10
(*a*)	0·25 cc.	7	2·1	0·022	9
(*b*)	0·35 cc.	9	2·5	0·027	8
Extract of rice polishings	0·25 cc.	5	1·6	0·0185	7
	0·5 cc.	10	5·1	0·0775	15
	1·0 cc.	10	5·7	0·098	10
	2·0 cc.	11	7·5	0·256	13
Yeast (soft extract I)	0·12 g.	3	1·7	0·019	16
	0·24 g.	6	3·5	0·039	16

except for (*a*) certain doses for which a relatively small number of animals was used, and (*b*) the highest dose of acid clay of which the result has to be interpreted from a very high, and therefore unreliable, part of the curve. The curve may, therefore, be considered a fairly reliable one for relating duration of cure to dose of vitamin B_1 given.

Moreover this curve and the curve relating percentage of birds to dose of vitamin B_1 give the same results within the limits of accuracy of the tests.

B. Method of calculating results by percentage of birds cured.

(*a*) *Standard deviation of the percentage number of birds cured.*

The percentage standard error of each of the 19 tests was calculated from the formula $\epsilon = \sqrt{\frac{p.q}{n}}$ where p = the percentage of pigeons cured, q = the percentage not cured, n = the total number of pigeons used in the test. From each value of ϵ the corresponding value of σ was calculated ($\sigma = \epsilon \sqrt{n}$). The weighted mean of the 19 values for σ was found to be 43·08 %.

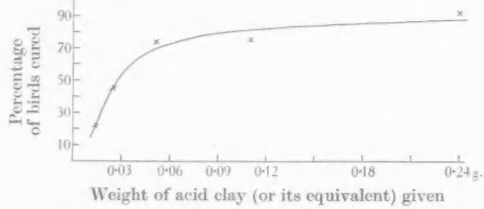

Fig. 8. Curve of response constructed from percentage of polyneuritic birds cured by doses of activated acid clay or the equivalent of these doses in other substances.

(*b*) *Relation of percentage of birds cured to dose of vitamin B_1 given.*

A curve of response (Fig. 8) has been constructed by grouping the pigeons and averaging results as in the last section. This curve is, therefore, based on more data than that in Fig. 2, it is extended through higher doses and it relates percentage of birds cured directly to the corresponding dose of the standard.

(*c*) *The degree of accuracy obtainable by this method.*

The degree of accuracy of an estimation by the use of 9 pigeons has been worked out as for the calculation of the duration of cure. It is summarised in Table XIII.

Table XIII. *The probabilities of obtaining certain degrees of accuracy in this test. Standard error of percentage of birds cured when 9 birds are used in a test.*

$$\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{43{\cdot}08}{\sqrt{9}} = 14{\cdot}36.$$

Percentage no. of pigeons cured, say,	Abscissa corresponding to % cured	Range of percentage number cured	Range of abscissae* corresponding to range of % number cured	Percentage range of abscissae
50	0·028	(*a*) $\pm\frac{2}{3}\epsilon = \pm 9{\cdot}57$ ($\frac{1}{2}$ chance)		
		59·57	0·036	129
		40·43	0·021	77
		(*b*) $\pm\epsilon = \pm 14{\cdot}36$ ($\frac{2}{3}$ chance)		
		64·36	0·042	150
		35·64	0·019	67
		(*c*) $\pm 2\epsilon = \pm 28{\cdot}72$ ($\frac{21}{22}$ chance)		
		78·72	0·084	300
		21·28	0·014	50

* Obtained by direct reading from curve of response.

It is evident from the figures in the last column of Table XIII that the curve relating the percentage of birds cured to dose of vitamin B_1 given is somewhat flatter at its upper end than the curve relating the duration of cure to dose of vitamin B_1 given. Except for results falling on the upper part of the curve, which one would try to avoid in either test, it is evident that equal degrees of accuracy are obtained in the estimation of vitamin B_1 by the use of polyneuritic pigeons in either way, provided the results are interpreted by means of curves of response such as we have constructed for this paper.

2. Rats.

The growth response.

(*a*) *The standard deviation of the response to a dose of vitamin B_1.*

The mean variance of the increase in weight in 3 weeks of rats given a dose of vitamin B_1 after they have ceased to grow on a diet deficient in this vitamin is 89·06 for bucks and 38·96 for does. Thus the standard deviation of the increase in weight in 3 weeks is 9·4 for bucks and 6·2 for does. These figures are presented with some hesitation as they were calculated from only 47 bucks and 36 does respectively. For purposes of calculating the degree of accuracy obtainable in the estimation of vitamin B_1 as carried out by the growth-promoting test in this laboratory, the two values for σ have been averaged ($M = 7{\cdot}8$).

(*b*) *The degree of accuracy obtainable by this test.*

If 7·8 be accepted as the value for σ then the standard error of the test using 9 rats on a dose is $\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{7{\cdot}8}{3} = 2{\cdot}6$. The degree of accuracy may then be calculated from the equation of the curve of response, $y = 139{\cdot}4 + 70{\cdot}4 \log x$ (Table XIV).

Table XIV. *Degree of accuracy obtainable with 9 rats on a dose.*

$\left[\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{7{\cdot}8}{3} = 2{\cdot}6\right]$.

Average increase in wt. in 3 weeks, say,	Abscissa corresponding to this increase	Range of increase in wt. in 3 weeks (g.)	Range of abscissae corresponding to range in increase in weight	% range of abscissae (doses)
20 g.	0·0201	(*a*) $\frac{2}{3}\epsilon = \pm 1{\cdot}74$ ($\frac{1}{2}$ chance)		
		21·74	0·0213	106·1
		18·26	0·0190	94·5
		(*b*) $\epsilon = \pm 2{\cdot}6$ ($\frac{2}{3}$ chance)		
		22·6	0·0219	108·6
		17·4	0·0186	92·6
		(*c*) $2\epsilon = \pm 5{\cdot}2$ ($\frac{21}{22}$ chance)		
		25·2	0·0239	118·9
		14·8	0·0170	84·5

Similarly the percentage range of abscissae, *i.e.* the percentage range of accuracy obtainable when only 4 rats are used on a dose of vitamin B_1 is:

1 in 2 chance (probable error)	92·6 to 108·6 %
2 in 3 chance	88·2 to 113·8 %
21 in 22 chance	77·6 to 129·5 %

Thus the degree of accuracy obtainable in an estimation of vitamin B_1 appears to be very high. As, however, the value for the standard deviation is based on only a small number of animals, it is possible that the degree of accuracy may not be as great as this, though it is unlikely that it will be very much less.

SUMMARY.

New methods are proposed for estimating the potency of preparations containing vitamin B_1 by means of (1) tests in which pigeons are cured of head retraction, (2) tests in which the growth of rats is observed. Several substances have been examined by these methods in comparison with the International Standard (I.S.).

The responses of pigeons (duration of cure and percentage of birds cured) and of rats (growth) to doses of vitamin B_1 are curvilinear. This fact must produce differences in results calculated by Peters's day-dose method according as the dose tested is high or low. With pigeons, the duration of cure and the percentage of birds cured give the same estimation of potency provided the results are interpreted by means of suitable curves relating response to dose of vitamin B_1 given.

The rat and pigeon methods give the same estimation of potency of a soft extract of yeast in comparison with the I.S. Thus the rat and pigeon appear to have equal ability for dealing with the vitamin B_1 in the Standard. In tests

of two different samples of dried yeast, however, the pigeon test indicated a vitamin B_1 potency about double that indicated by the rat test. Thus the rat appears to be less able to respond to the vitamin B_1 of dried yeast than does the pigeon. Statistical estimations of the results indicated that the probable errors of the tests using 9 animals in a test are:

(1) pigeon, (*a*) duration of cure ... ... +31 or −26 %,
(*b*) percentage cured ... ... +29 or −23 %;
(2) rat, mean increase in weight in 3 weeks ... + 6·1 or − 5·5 %.

Moreover, the standard deviation of results obtained by pigeons is no greater in tests on solids (*e.g.* acid clay or dried yeast) than it is for liquids (*e.g.* extracts of yeast). Therefore, there is apparently no greater variation in the ability of different birds to deal with solids than in their ability to deal with liquids.

In spite of the probable error of the pigeon test being much greater than that of the rat test, the former has the great advantage of being specific for the factor it is used to estimate. It may also be that it gives a truer result with certain substances (*e.g.* dried yeast) than the rat test, though it may be difficult to use the polyneuritic pigeon for testing many substances which cannot be suspended in water and given by a stomach tube.

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